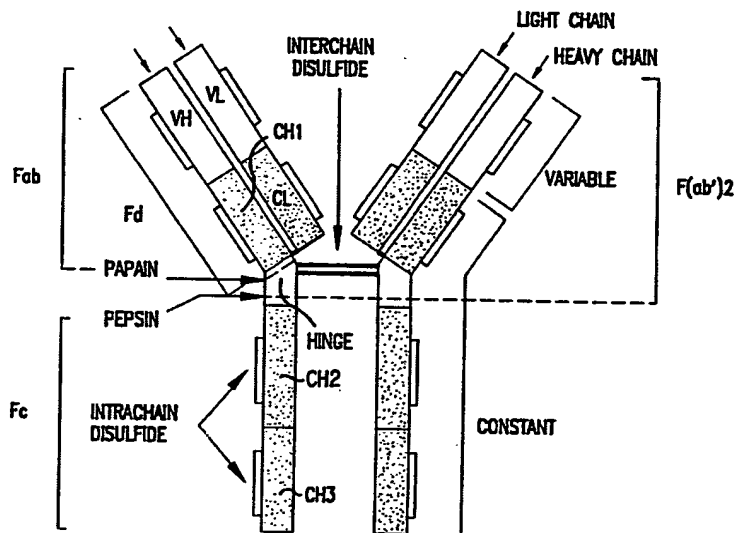




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<p>(21) International Application Number: PCT/US92/04976</p> <p>(22) International Filing Date: 15 June 1992 (15.06.92)</p> <p>(30) Priority data: 714,175 14 June 1991 (14.06.91) US</p> <p>(71) Applicant: XOMA CORPORATION [US/US]; 2910 Seventh Street, Berkeley, CA 94710 (US).</p> <p>(72) Inventors: HORWITZ, Arnold, H. ; 7529 Midfield Avenue, Los Angeles, CA 90045 (US). BETTER, Marc, D. ; 2462 Zorada Drive, Los Angeles, CA 90046 (US). CARROLL, Steve ; 1308 Miltar Avenue, Walnut Creek, CA 94596 (US).</p>		<p>(74) Agents: GOLDSTEIN, Jorge, A. et al.; Sterne, Kessler, Goldstein &amp; Fox, 1225 Connecticut Avenue, N.W., Suite 300, Washington, DC 20036 (US).</p> <p>(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent).</p> <p><b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>

(54) Title: MICROBIALY-PRODUCED ANTIBODY FRAGMENTS AND THEIR CONJUGATES



## (57) Abstract

Recombinant DNA methods are described for the production of antibody  $F(ab')_2$  and  $Fab'$  fragment molecules from transformed microorganisms such as bacteria and yeast. The recombinant  $F(ab')_2$  and  $Fab'$  fragments are secreted from the host cells and may be recovered directly from the fermentation culture. These direct methods of  $F(ab')_2$  and  $Fab'$  production are advantageous over existing art methods by avoiding proteolytic digestions and obtaining homogeneous molecules. Methods are described for the conjugation of these fragments, by methods targeting either available lysine amino acid residues or selectively-reducible cysteine residues, to active moieties such as ricin toxin A chain. The microbially-produced  $F(ab')_2$  and  $Fab'$  fragment molecules and their immunoconjugates have a wide variety of therapeutic and diagnostic uses.

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## TITLE OF THE INVENTION

MICROBIALY-PRODUCED ANTIBODY  
FRAGMENTS AND THEIR CONJUGATES

## BACKGROUND OF THE INVENTION

### Field of the Invention

This invention relates to methods for producing antibody  $F(ab')_2$  and Fab' fragments by recombinant DNA manipulations and microbial fermentation, and methods for their use in the preparation of protein conjugates, including immunoconjugates.

### Brief Description of the Background Art

The development of monoclonal antibody technology has resulted in the proliferation of antibodies for use in research, diagnostic and therapeutic applications. Many of these antibodies originate from mouse hybridomas. Such non-human antibodies can be problematic for use as *in vivo* diagnostics and as therapeutic agents in humans due to immunogenicity. This is an especially important consideration when these products are used for repeated treatment over extended periods of time. One solution to the problem of mouse antibody immunogenicity is to use recombinant DNA technology to produce mouse-human chimeric antibodies in which gene sequences encoding mouse V regions are fused to gene sequences encoding human constant regions (reviewed by

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Morrison *et al.*, *Adv. Immunol.* 44: 65-92 (1989)). In addition to partially "humanizing" the mouse-derived antibody, the same genetic engineering technology used for producing chimeric antibodies can also be used to permit easy class switching (Shaw *et al.* *J. Natl. Cancer Institute* 80: 1553 (1988)).

Concurrent with the production and engineering of monoclonal antibodies directed towards a variety of tumor antigens has been the development of technologies for the use of these antibodies for *in vivo* diagnosis of tumor metastases (*i.e.*, tumor imaging) and for therapy, either as naked antibodies or coupled with therapeutically efficacious agents such as radionuclides, drugs and toxins. However, the whole antibody, whether it be all mouse, or mouse-human chimeric, may have disadvantages for certain diagnostic and/or therapeutic applications where greater tumor penetration, less liver interaction, or more rapid clearance time is desired. Instead, antibody fragments such as Fab, Fab' and F(ab')<sub>2</sub>, which consist of the antigen-binding subunits but lack regions containing the effector functions (Fc), have been demonstrated to be efficacious in animal models (Colapinto *et al.* *Cancer Res.* 48: 5701 (1988); Wahl *et al.* *J. Nucl. Med.* 24: 316 (1983)), and in human clinical studies (Delaloye *et al.* *J. Clin. Invest.* 77: 301 (1986)). These fragments, which are generated by proteolytic digestion of whole antibody, have been shown to give higher tumor/blood ratios for imaging and promote deeper penetration into tumors for therapeutic applications than whole antibody.

F(ab')<sub>2</sub> molecules, which consist of two Fab units linked by the interchain disulfide bonds in the hinge region, retain the binding affinity achieved by the whole antibody. Unconjugated F(ab')<sub>2</sub> molecules may be useful therapeutic agents and have been shown to have equal or higher efficacy as whole antibodies in immunosuppression models (Carteron *et al.* *Clin. Immuno. Immunopath.* 56: 373-383 (1990), and Hirsch *et al.* *Transplantation Proceedings* 23: 270-271 (1991)). Furthermore,

immunoconjugates of  $F(ab')_2$  may be superior to those containing whole antibodies for imaging applications or for delivery of therapeutic agents to tumors (Colapinto, *supra*; Delaloye, *supra*; Wahl, *supra*).

$F(ab')_2$  molecules are also useful since the individual antigen-binding units (Fab') can be separated by using mild reducing conditions (Johnstone *et al. Immunochemistry in Practice* (Blackwell, Oxford), page 52 (1982)). The resulting Fab' fragments contain reactive sulfhydryl groups which can be used for covalent attachment of label, or other clinically or experimentally useful molecules. They also can serve as the starting point for the construction of heterobifunctional  $F(ab')_2$  molecules consisting of individual Fab' units with different specificities. Examples of such heterobifunctional  $F(ab')_2$  molecules include those which combine specificity toward a tumor antigen with a chelating agent for a radionuclide, or toward an antigen and an Fc receptor.

A number of clinically important antibodies, or their  $F(ab')_2$  and Fab fragments, have been used as immunoconjugates with cytotoxic organic compounds, protein toxins, and radionuclides (Yang *et al. Proc. Natl. Acad. Sci. USA* 85: 1189 (1988); Blakey *et al. Prog. Allergy* 115: 50 (1988); Delaloye *et al., supra*). Typically, the crosslinking reagents are coupled to the lysine residues of the antibody molecules which have been first derivatized with bifunctional cross-linking reagents such as N-succinimidyl-3 (2-pyridyldithio)propionate (SPDP), which modified lysine residues throughout the molecule.

We have recently described two systems for producing genetically engineered Fab fragments by secretion from *Saccharomyces cerevisiae* (Horwitz *et al. Proc. Natl. Acad. Sci. USA* 85: 8678-8682 (1988)) and *Escherichia coli* (Better *et al. Science* 240: 1041-1043 (1988)). While microbially-produced Fab molecules are useful as starting points for the development of certain reagents, they do not retain the same degree of

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binding affinity of the whole antibody or  $F(ab')_2$ . Furthermore, Fab fragments lack the specific thiol conjugation properties of Fab'.

Current methods for  $F(ab')_2$  production involve pepsin digestion of whole antibody (Johnstone, *supra*, at 53-55). This approach can be problematic for several reasons. First, not all antibodies are equivalent in their susceptibility to pepsin cleavage. Second, digestion with pepsin can result in partial degradation of the fragments which may not be apparent when analyzed in non-reducing gels. Finally, additional purification steps are required beyond that for the whole antibody.

Approaches to the direct production of  $F(ab')_2$  molecules have been taken with engineered animal cells. (Neuberger *et al. Nature* 312: 604 (1984)) were able to obtain production of a  $F(ab')_2$ -like fragment by modification of the Fd' gene to contain a 21-amino-acid tailpiece from an immunoglobulin secretion exon. Bodmer *et al. (WO 8901783 (1989))*, reported obtaining only Fab' from COS cells, which could be subsequently associated to form  $F(ab')_2$ . Also, Gillies *et al. (Hum. Antibod. Hybrid. 1: 47 (1990))*, failed in their attempt to directly make  $F(ab')_2$  fragments from animal cells. Given the difficulties associated with their production in animal cells, it is unpredictable as to whether functional  $F(ab')_2$  molecules could be directly made in a properly folded and assembled state by microorganisms such as bacteria or yeast.

Fab' fragments are useful for thiol-directed conjugation, and may be generated by selective reduction of  $F(ab')_2$  fragments (Johnstone, *supra*, at 53-55). Fab' produced by selective thiol reduction of  $F(ab')_2$  may however have the same problems mentioned above for  $F(ab')_2$  generated by classical enzymatic methods.

There is therefore a substantial need for the establishment of methods for the direct production of functional  $F(ab')_2$  and Fab' molecules from microorganisms. There is also a need to establish conditions for their use in the generation of effective immunoconjugates.

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The present invention demonstrates that, surprisingly, microorganisms can secrete such antibody fragments. Methods are described for their use in the production of active  $F(ab')_2$  or  $Fab'$  immunoconjugates and heterobifunctional  $F(ab')_2$  molecules.

### SUMMARY OF THE INVENTION

This invention provides immunoglobulin fragments, such as  $F(ab')_2$ , which retain the full binding affinity of whole antibody. This invention also provides immunoglobulin fragments, such as  $Fab'$ , which have the useful feature of convenient and selective thiol conjugation to polypeptide and chemical moieties. These fragments are produced directly from microorganisms, such as bacteria and yeast, which are engineered to secrete intact recombinant  $F(ab')_2$  ( $rF(ab')_2$ ) and recombinant  $Fab'$  ( $rFab'$ ) fragments. This production system is advantageous over traditional proteolysis methods which have additional steps and often give nicked or partially degraded antibody fragments. Various gene constructs are detailed which show truncated versions of a chimeric, mouse variable-human constant, immunoglobulin IgG1 heavy chain gene ( $Fd$ ) encoding 1 or 2 cysteine residues to the carboxyl side of the light chain- $Fd$  chain disulfide bond. These modified genes were assembled into vectors and transformed into either bacteria or yeast that also expressed the homologous chimeric light chain gene. Intact  $F(ab')_2$  and  $Fab'$  molecules were recovered by purification from fermentation broths.

Methods are provided for the conjugation of microbially-produced  $F(ab')_2$  molecules with protein toxins such as ricin toxin A chain to form immunotoxins. These immunotoxins are advantageous in that they have reduced levels of heterogeneity caused by proteolytic nicks introduced by standard art procedures for  $rF(ab')_2$  generation. These immunotoxins are also advantageous because they provide high affinity binding and the

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rF(ab')<sub>2</sub> molecules lack Fc receptors which may cause non-specific uptake by macrophages and other cells of the immune system.

The purified rF(ab')<sub>2</sub> and rFab' fragments from microbial fermentation have blocked cysteine thiol groups, which required the discovery of methods to unblock the cysteines for Fab' thiol conjugation. The invention provides reducing agents and conditions that achieve the selective reduction of the cysteine residue(s) nearest the carboxy terminus of Fd, without reducing the interchain disulfide linkage of light chain to Fd. These reduced Fab' fragments are conjugated to other proteins, polypeptides, or chemical moieties reactive with the free thiol group.

The invention provides methods for the conjugation of a reduced Fab' fragment by mixing it with a second polypeptide which contains free thiols under sufficient oxidizing conditions to form a disulfide linkage. The examples show that reduced Fab' fragments can be successfully conjugated to similarly reduced Fab' fragments to form either homodimeric or bifunctional, heterodimeric F(ab')<sub>2</sub> molecules. Other useful proteins containing free thiol groups can be similarly conjugated to the Fab' fragments to form mixed-function molecules.

The invention also provides methods for the chemical modification of reduced, microbially-produced Fab' fragments to achieve directed conjugation of the activated immunoglobulin fragment. A free thiol of the reduced Fab' fragment is reacted with activating moieties such as dithiobis(pyridine-N-oxide), and the activated Fab' is mixed with a polypeptide containing a free thiol. This results in efficient conjugation to form mixed-function molecules, such as a Fab'-ricin toxin A molecule, or a bifunctional heterodimer F(ab')<sub>2</sub> molecule.

### BRIEF DESCRIPTION OF FIGURES

Figure 1(a) is a physical representation of the structure of IgG. Shown are the positions of endopeptidase cleavages for papain and pepsin used to generate  $F(ab')_2$  and Fab fragments, respectively. The position of both intrachain and interchain disulfide bonds are shown, as are the protein domains VH, CH1, CH2, and CH3 on the heavy chain and VL and CL on the light chain.

Figure 1(b) represents the DNA and corresponding peptide sequences of the Fd' modules hinge region used for the production of  $F(ab')_2$  and Fab'.

Figure 2 represents the construction scheme for modules containing gene sequences encoding Fd' with one or both inter-heavy chain cysteines. Not drawn to scale.

Figure 3 represents the construction scheme for the gene module encoding Fd' with two inter-heavy chain cysteines plus 29 amino acids. By this construction scheme, the carboxy terminal amino acid is an Asp (Glu is normally at this position). Not drawn to scale.

Figure 4 represents the construction scheme for optimized yeast expression plasmids containing the ING-4 chimeric light chain and various Fd' genes fused to the PGK promoter (P), invertase signal sequence (S), and PGK polyadenylation signal (T). Not drawn to scale.

Figure 5 represents the binding inhibition of yeast-derived ING-4  $F(ab')_2$  and of ING-4  $F(ab')_2$  generated by pepsin digestion of ING-4 IgG. The yeast and pepsin-generated ING-4  $F(ab')_2$ , as well as ING-4 Fab and IgG, were used to inhibit binding of biotinylated ING-4 IgG to the surface of antigen-positive HT29 colon carcinoma cells. Biotinylated IgG was incubated with HT29 tumor cells in the presence of competing antibody at 4°C. Cells were washed and further incubated with avidin-peroxidase at room temperature. The cell-bound peroxidase was

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visualized with OPD reagent, and its OD490 was used to determine the extent of inhibition.

Figure 6 represents the construction scheme for bacterial expression plasmid containing the gene sequences encoding the H65 chimeric light chain and Fd' chain with one or two inter-heavy chain cysteines. Each gene was fused to the *E. carotovora pelB* ribosome binding site and signal sequence. These were fused to each other and placed under the control of the *Salmonella typhimurium araBAD* promoter and the *trp* transcription termination sequence in a plasmid containing tetR gene for selection in *E. coli*. Not drawn to scale.

Figure 7 represents the SDS polyacrylamide gel analysis of various chimeric H65 Fab and Fab' molecules secreted from bacteria. Regions of the gel which were scanned by densitometry are denoted.

Figure 8 represents the DNA sequence of pXOM1 (H65 VH). Shown is the nucleotide sequence including the ATG initiation codon to the JK/CK junction. Also shown is the predicted amino acid sequence of the region. Shown in bold are the regions where PCR primers bound for amplification of the V-J region.

Figure 9 represents the DNA sequence of pXOM2 (H65 VL). Shown is the nucleotide sequence including the ATG initiation codon to the JK/CK junction. Also shown is the predicted amino acid sequence of the region. Shown in bold are the regions where PCR primers bound for amplification of the V-J region.

Figure 10 represents the DNA sequence of the 4A2 kappa V-region. Shown is the nucleotide sequence for the ATG initiation codon to the JK/CK junction. Also shown is the predicted amino acid sequence of the region. Shown in bold are the regions where PCR primers bound for amplification of the V-J region.

Figure 11 represents the DNA sequence of the 4A2 gamma V-region. Shown is the nucleotide sequence for the ATG initiation codon

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to the JH/CH junction. Also shown is the predicted amino acid sequence of the region. Shown in bold are the regions where PCR primers bound for amplification of the V-J region.

Figure 12 represents the construction scheme for bacterial expression plasmid containing the gene sequences encoding the 4A2 chimeric light and Fd' chains. Each gene was fused to the *E. carotovora pelB* ribosome binding site and signal sequence. These were fused to each other and placed under the control of the *Salmonella typhimurium araBAD* promoter and the *trp* transcription termination sequence in a plasmid containing *tetR* gene for selection in *E. coli*. Not drawn to scale.

Figure 13 represents the cytotoxicity mediated by ricin A chain immunoconjugates prepared from H65 antibodies and fragments. The human T cell line HSB2 was exposed to H65 mouse antibody conjugated to ricin toxin A (RTA) chain (-o-), or chimeric H65 Fab', either unconjugated (-Δ-) or conjugated (-▽-) to ricin toxin 30-kd A (RTA30) chain. Cell viability was determined by the percent of leucine incorporated into acid-precipitable radioactivity.

Figure 14 represents the cytotoxicity of resting (panel a) and phytohemagglutinin-activated (panel b) human peripheral blood mononuclear cells, mediated by various H65 antibodies and fragment immunoconjugates. The samples tested were H65 mouse antibody linked by 5-methyl-2-iminothiolane to RTA30 (-o-), and chimeric H65 Fab' linked to RTA30 (-□-). IND2 antibody linked to RTA30 (-Δ-) and RTA30 alone (-◇-) were included as additional controls.

#### DEFINITIONS

The following definitions are supplied in order to provide clarity in the description of the invention.

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By the term "V domain" is intended the variable region polypeptide sequence of an immunoglobulin light chain, as shown by Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 4th ed. (U.S. Dept. of Health and Human Services, NIH) (1987).

By the term "CL domain" is intended the constant region polypeptide sequence of an immunoglobulin light chain, as shown by Kabat *et al.*, *supra*.

By the term "CH1 domain" is intended the first constant region polypeptide sequence of an immunoglobulin heavy chain that is carboxy to the V domain, as shown by Kabat *et al.*, *supra*.

By the term "hinge domain" is intended the constant region polypeptide sequence of an immunoglobulin heavy chain that is on the carboxyl side of the CH1 domain, as shown by Kabat *et al.*, *supra*.

By the term "rFab" is intended an antigen-binding immunoglobulin fragment or its equivalent containing an intact light chain and a truncated heavy chain, linked by an interchain disulfide bond, and which includes at least one cysteine residue in the hinge domain which is carboxy to the light chain-Fd interchain disulfide bond.

By the term "Fab'" is intended recombinantly produced Fab'.

By the term "F(ab')<sub>2</sub>" is intended a dimer of Fab' molecules linked by at least one disulfide bond involving a cysteine residue in the hinge domain which is carboxy to the light chain-Fd interchain disulfide.

By the term "rF(ab')<sub>2</sub>" is intended recombinantly produced F(ab')<sub>2</sub>.

By the term "Fv" is intended an antigen-binding immunoglobulin fragment or its equivalent containing only the V domains of light and heavy chains.

By the term "Fd'" is intended the heavy chain of a Fab' molecule.

By the term "RTA" is intended ricin toxin A chain.

By the term "RTA30" is intended the Mr 30,000 form of ricin toxin

A.

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Recognizing that conventional amino acid numbering is from left to right; and that the amino terminus is conventionally shown on the left, with the carboxyl terminus on the right; the term "cysteine residue with the highest residue number" is the cysteine residue with the highest amino acid number, or, stated another way, the cysteine closest to the carboxy terminus.

By the term "thiol-containing active moiety" is intended immunoglobulin Fab' molecules, enzymes, polypeptides, radionuclides, and organic or inorganic compounds containing a reactive sulfhydryl group.

By the term "culture medium" is intended a nutritive solution for culturing or growing cells. The ingredients that compose such media may vary depending on the type of cell to be cultured. In addition to nutrient composition, osmolarity and pH are considered important parameters of culture media.

By the term "tumor-associated antigen" is intended a tumor bearing antigen(s) recognized by the Fab' or F(ab')<sub>2</sub> of the present invention. Specific examples of tumor associated antigens are disclosed in European Patent Application Number 8730600.

#### DESCRIPTION OF PREFERRED EMBODIMENTS

Various genes which encode immunoglobulin fragments are provided by the present invention. The preferred genes encode both light and heavy chains, and retain complete variable regions for the light and heavy chains to provide at least an active immunoglobulin Fv binding domain. In addition, the heavy and light chain genes encode additional peptide sequences which have at least one cysteine residue which is not located within the immunoglobulin Fv binding domain. These peptide sequences are preferably a CL (kappa or lambda) region for light chain,

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and a CH1 and hinge region for Fd chain. The genes are preferably linked to a secretion signal appropriate for the host, such as the pectate lyase B signal peptide for bacterial hosts, or the yeast invertase signal peptide for yeast hosts.

A preferred embodiment of the invention is a host transformed with a complete light chain gene and a truncated heavy chain gene (Fd) which encode an Fab' fragment molecule. These molecules are capable of spontaneous assembly into  $F(ab')_2$  fragments. The gene sequences encoding light chain CL and Fd chain CH1 and hinge domains may be derived from either human or non-human immunoglobulins of any isotype. For *in vivo* human uses, human CL, CH1, and hinge domains are preferred; they are more compatible with the human body than non-human domains. The invention exemplifies the use of human IgG1 isotype sequences as the source of CH1 and hinge domains. For the human IgG1 isotype, purification of homogeneous  $F(ab')_2$  or Fab' molecules from microbial fermentations is enhanced by including more than one, preferably two, cysteine residues in the Fd hinge region on the carboxyl side of the light chain Fd disulfide bond. A polypeptide tail may follow on the carboxyl side of the two cysteine residues.

Other immunoglobulin fragments may be generated within the practice of this invention. For example, a selectively reducible cysteine residue may be located on the light chain of an Fab-like molecule by adding a polypeptide sequence to the carboxy terminus of the light chain. Another embodiment of the invention is the modification of the heavy chain gene so that a partial deletion is used to generate a modified immunoglobulin, containing at least an Fab region, and having a selectively reducible cysteine residue in the hinge domain. Another embodiment of the invention is the modification of Fv fragment genes by including sequences encoding an additional polypeptide region (or regions) on either the light or heavy chain (or both), with such additional

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polypeptide (or polypeptides) encoding a selectively reducible cysteine residue (or residues).

The  $F(ab')_2$  and Fab' fragments of the present invention may be produced from a variety of host cells. Preferred hosts are bacteria and yeast. Vectors are exemplified for the bacterium *E. coli*, and the yeast *S. cerevisiae*. Other hosts may be utilized in the practice of this invention, including gram-negative bacteria such as *Salmonella typhimurium* or *Serratia marcescens*, and various *Pseudomonas* species; gram-positive bacteria; other yeasts and fungi, and plant, insect, and animal cells. Preferable features for hosts include the ability to be practically grown in industrial fermenters and bioreactors, and the capability of the secretion of intact immunoglobulin fragments. A method for the preparation of  $F(ab')_2$  and Fab' fragments is the culturing of host cells on culture media followed by isolation of active fragment from the fermentation broth, preferably after removal of cells from the broth.

Reducing agents useful for the selective reduction of the cysteine residues used for conjugation include dithiothreitol, cysteine, beta-mercaptoethanol, and the like. As shown in the examples, different concentrations of reducing agents may be required to achieve the desired selective reduction. For  $F(ab')_2$  molecules secreted from bacteria or yeast where the Fab' molecules are joined by a single disulfide bond, 2.0 mM cysteine selectively reduces the single heavy chain-heavy chain disulfide and is preferred. However, 2.0 mM cysteine is insufficient to reduce two hinge region heavy chain-heavy chain disulfide bonds, which require approximately 5.0 to 15.0 mM cysteine for selective reduction.

For Fab' molecules secreted from bacteria or yeast, the desired hinge region cysteine residue (or residues) are blocked by small molecular weight adducts. These residues may be unblocked by reduction with dithiothreitol at concentrations of about 0.1 to 2.0 mM, or by cysteine at concentrations of about 5.0 to 15.0 mM.

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The selectively-reduced Fab' fragments can be conjugated to useful thiol-containing moieties such as enzymatic and non-enzymatic polypeptides, Fab' fragments, radionuclides, and other compounds. In one method, selectively-reduced Fab' molecules are placed in oxidizing conditions to form disulfide-linked  $F(ab')_2$  fragments. In a second method, the Fab' molecule is reacted by disulfide exchange with an activating compound such as dithiobis(nitrobenzoate), dithiobis (pyridine-N-oxide) or the like, creating an excellent leaving group for directed disulfide formation. The activated Fab' is then reacted with a thiol-containing moiety for the formation of heteroconjugates such as a heterobifunctional  $F(ab')_2$  or a Fab'-enzyme.

An alternate method for heteroconjugate formation is the reaction of the reduced Fab' molecule with linker compounds which have functional groups reactive with Fab' thiols such as maleimide and the like, to form a Fab'-linker conjugate. The Fab'-linker conjugate is then reacted with a useful active moiety to form a heteroconjugate. Generally, any sulfhydryl linking compound may be utilized. For example, the linker compound may have an S-acetyl functional group. The Fab-linker, S-acetyl, is reacted with a thiol-containing enzyme or other polypeptide which has been activated with an appropriate compound, such as dithiobis (nitrobenzoate) or dithiobis(pyridine-N-oxide). Such a heteroconjugate has a disulfide linkage to the active moiety. For Fab'-enzyme heteroconjugates with cytotoxic enzymes such as ricin A chain, a disulfide linkage is preferred for maximal cytotoxic activity. For other uses, a linker compound which forms other than a disulfide linkage to the active moiety may be used. Such uses include, for example, a thioether linkage to enhance stability of a Fab'-conjugate.

Both homodimeric and heterodimeric  $F(ab')_2$  molecules are provided by the invention. Homodimeric  $F(ab')_2$  are preferred when a single specificity with bifunctional binding is desired. Bispecific,

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heterodimeric  $F(ab')_2$  fragments are preferred when the separate binding functions of heterobifunctional antibodies are desired. The heterodimeric  $F(ab')_2$  are preferred over known bispecific antibodies in their properties of a smaller molecular weight and the deletion of the Fc region, which can be advantageous when better tissue penetration and minimization of Fc-receptor cell interactions are desired. There are different methods provided by this invention for the formation of heterodimeric  $F(ab')_2$  molecules. In one method,  $F(ab')_2$  or Fab' with different specificities are first reduced to monovalent Fab' forms. They are then either mixed and oxidized to form heterodimeric  $F(ab')_2$ , or are reacted with linker compounds known in the art and subsequently mixed and reacted to form  $F(ab')_2$ . Another method is to use the hosts and vectors of this invention to separately express the genes encoding the two different Fab' molecules within the same host cell. Heterodimeric  $F(ab')_2$  may then be purified from the fermentation culture.

Enzymes may be conjugated to microbially-produced  $F(ab')_2$  and Fab' molecules to form immunoconjugates with therapeutic or diagnostic use. Examples of therapeutically useful enzymes include protein toxins, such as the ribosome-inhibitor ricin A chain, to achieve therapeutically targeted killing of cells, and other enzymes such as alkaline phosphatase, to achieve therapeutic effects by prodrug conversion into active drug, Pseudomonas toxin, Diphtheria toxin, and Tumor Necrosis Factor (TNF). Such  $F(ab')_2$  or Fab'-enzyme conjugates can also be used in *in vitro* diagnostic assays to convert a substrate into a detectable form, in a similar way as immunoassays known in the art.

The  $F(ab')_2$  fragments or activated Fab' fragments of the invention may be conjugated to non-enzymatic polypeptides that interact with cellular receptors, such as interleukin-2, epidermal growth factor, immunoglobulin Fc regions, and the like. Such molecules could be useful in activating cells to accomplish desired effector functions, such as the

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selective activation or killing of targeted cells, and thereby achieve a therapeutic effect.

F(ab')<sub>2</sub> fragments may be conjugated to enzymes and other polypeptides by current art methods that rely on the derivatization of amino acid residues with linker compounds such as N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), or preferably sterically hindered linkers such as the substituted 2-iminothiolanes (Goff *et al.*, *Bioconjugate Chem.* 1: 381-386 (1990)). Other examples of linker compounds may be found in U.S. Patent No. 4,970,303. The derivatized F(ab')<sub>2</sub> is then reacted with a thiol-containing polypeptide to form a stable conjugate.

The number and placement of polypeptides or enzymes which may be conjugated to the cysteine thiol(s) of a Fab' molecule is limited by the number and placement of selectively reducible cysteine residues. The preferred location of cysteines is away from the variable regions which define and accomplish the Fab' binding activity. The number of selectively reducible cysteines is from 1 to about 10, preferably 2, to achieve targeted conjugation and avoid interference of the enzymatic activity with the binding activity.

A functionally derivatized F(ab')<sub>2</sub> or a selectively-reduced Fab' may also be conjugated to chemical moieties (radionuclides and organic compounds) that confer a desired second function on the Fab'. Such chemicals include cytotoxic compounds such as trichothecenes, and metal-chelating compounds such as diethylene triamine pentaacetic acid (DTPA), daunorubicin, doxorubicin, methotrexate, Mitomycin C and others that are known in the art. See Goodman *et al.*, Goodman and Gilman's THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, 7th Ed., Macmillan Publishing Co., (1983). Examples of radionuclides include <sup>212</sup>Bi, <sup>131</sup>I, <sup>186</sup>Re, and <sup>90</sup>Y, which list is not intended to be exhaustive. Also, a selectively-reduced Fab' may be directly bonded to

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certain diagnostic or therapeutic radionuclides, such as  $^{99}\text{Tc}$  or  $^{186}\text{Re}$ , by methods that are known in the art.

It is further recognized that in-frame fusions, between the 3' end of the Fd or Fd' gene and other useful sequences may be constructed.

Having generally described the invention, a further understanding can be obtained by reference to certain specific examples which are provided herein for the purpose of illustration only and are not intended to be limiting unless otherwise specified.

### EXAMPLES

#### Example 1: Construction of Fd' Gene Modules

Cellular production of  $\text{F(ab')}_2$  or Fab' molecules requires the co-expression of genes encoding the light chain and a heavy chain fragment designated as Fd'. The human IgG1 Fd' fragment contains at least one of the two cysteines involved in heavy chain interchain disulfide bond formation (Figure 1a).

Six different Fd' gene modules were constructed. The DNA and corresponding peptide sequences at the carboxy terminus of these modules are shown in Figure 1b. The first of these (Figure 1b-A), encoding an Fd' with a single inter-heavy chain cysteine was constructed as outlined in Figure 2, generating the plasmid pING1624. This construction makes use of the fact that the Fd gene (on pING1412) previously engineered for Fab production contains a unique *Bcl*I site at the stop codon which had been introduced in the DNA sequence encoding the first of two interchain cysteines. Consequently, digestion of pING1412 with *Bcl*I, followed by treatment with mung bean nuclease, leaves a blunt end consisting of a TA base pair. Fusion of the linker:

5'-GTCCACCATGATCACTCGA-3'

## 3'-CAGGTGGTACTAGTGAGCT-5'

to this blunt end regenerates the codon (TGT) for the first of two cysteines in hinge plus the codons (CCA) for the two prolines that follow. Association of this Fd' with a light chain will result in a Fab' molecule with a single Cys carboxy to the light chain-Fd disulfide or a F(ab')<sub>2</sub> with a single disulfide bond between the two Fab' moieties. As with the construction of the Fd gene module for Fab production, a *Bcl*I site was incorporated at the stop codon to facilitate subsequent gene fusions.

Two Fd' modules (Figure 1b-B and C) were constructed using pING1624 as a starting point and the same strategy used for construction of pING1624. These modules contained both of the cysteine codons for heavy chain-interchain disulfide bond formation plus codons for either two or nine additional amino acids 3' to the cysteine codons (Figure 1) and were designated pING1673 and pING1684, respectively (Figure 2c).

A fourth Fd' module (Figure 1b-D) was constructed as outlined in Figure 3. This module (pING1695, Figure 3c) encodes both inter-heavy chain cysteines plus 29 additional amino acids 3' to the cysteines.

Two additional Fd' modules (Figure 1b-E and F) were also constructed. In these modules, the first of two inter-heavy chain cysteines was changed to serine by site-directed mutagenesis. These novel modules were constructed as part of an attempt to decrease the heterogeneity of Fab' molecules containing a single interchain tail and secreted from microorganisms, as described in Example 3. All of the above Fd' gene modules contain unique *Bcl*I sites at the stop codon.

The approaches described above can be generally applied to accomplish the construction of in-frame fusions between the 3' end of the Fd or Fd' gene modules and other useful gene sequences. Examples of such constructions include various other types of truncated heavy chain modules and fusions between Fd or Fd' and a variety of molecules of diagnostic, therapeutic or experimental importance, such as

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immunoglobulin heavy chain domains, receptor-binding proteins, or cytotoxic polypeptides.

Example 2: Secretion of Functional F(ab')<sub>2</sub> and Fab' by Yeast

Yeast cells are capable of secreting functional Fab and IgG molecules such as mouse-human chimeric IgG and Fab molecules (Horwitz *et al.*, *supra*). Secretion of IgG is accomplished by co-expressing genes encoding the mature forms of light chain and heavy chain each fused to yeast invertase signal sequence and PGK promoter and polyadenylation signals. Fab secretion is accomplished by co-expressing light chain with a genetically engineered Fd chain containing a stop codon introduced at the gene sequences encoding the first of two cysteines responsible for IgG1 heavy chain interchain disulfide bonds. As for the light chain gene, the Fd chain gene is fused to the yeast invertase signal sequence-PGK promoter and polyadenylation signal.

In the present example, yeast serves as a host for the production of mouse-human chimeric F(ab')<sub>2</sub> and Fab' molecules. The chimeric antibody fragments in this example contain the light and heavy chain variable regions of a monoclonal antibody designated Me4, which binds to an antigen expressed on the surface of cells from many melanomas and carcinomas. The chimeric version of Me4 is designated as ING-4, and the production of ING-4 IgG1 by mouse Sp/20 cells and of Fab by yeast and bacterial cells have been previously described (Better *et al.*, PCT US8903852).

a. Yeast strains and growth conditions

*S. cerevisiae* strain PS6 (*ura3 leu2 MATa*) was developed and subsequently used as a host for yeast transformations performed as

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described by Ito *et al.* *J. Bacteriol.* 153: 163-168 (1983). Yeast transformants were selected on SD + leu agar (2% glucose, 0.67% yeast nitrogen base, 2% agar) and grown in SD + leu broth buffered with 50 mM sodium succinate, pH 5.5.

b. Construction of yeast expression plasmids containing antibody genes

Production of F(ab')<sub>2</sub> or Fab' molecules by yeast requires the simultaneous production of both the light chain and Fd' chain proteins. This can be accomplished by co-transforming a leu2- and ura3- strain with plasmids containing the light and Fd' chain genes with the leu2 and ura3 genes, respectively. Optimal production is achieved by placing both the light and Fd chain genes on the same plasmid. Accordingly, the Fd' gene module from pING1624 (module A, Figure 1) described in Example 1 was ligated to a ura3 expression vector to form plasmid pING1636 (Figure 4b). The yeast expression plasmid pING1697 was then constructed as shown in Figure 4c. pING1697 contains the gene sequences encoding the mature forms of the ING-4 chimeric light chain plus the chimeric Fd' chain module A gene fused to the DNA sequences encoding the invertase signal sequence and the PGK promoter and polyadenylation signals. Using this method, the plasmids pING1673, pING1684 and pING1695 were used to construct expression plasmids pING1698 (module B), pING1800 (module C), and pING1699 (module D).

c. Yeast secretion of chimeric ING-4 F(ab')<sub>2</sub> and Fab'

The plasmids pING1697, pING1698, pING1699, and pING1800 were transformed into *S. cerevisiae* PS6 by selection for Ura<sup>+</sup> colonies in SD<sup>+</sup> leu agar. Transformants were grown in SD broth lacking uracil and the levels of secreted F(ab')<sub>2</sub> or Fab' assessed by ELISA. The cultures

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secreting the highest levels of Fab' (pING1697, 0.6  $\mu\text{g/ml}$ ; pING1698, 1.9  $\mu\text{g/ml}$ ; pING1699, 1.9  $\mu\text{g/ml}$ ; pING1800, 2.5  $\mu\text{g/ml}$ ) were grown in 10 liters of SD broth for 60 hours and  $\text{F(ab')}_2$  and Fab' proteins were purified from the culture supernatant.

d. Isolation of chimeric  $\text{F(ab')}_2$  and Fab' from yeast and production of  $\text{F(ab')}_2$  from chimeric IgG

A mixture of  $\text{F(ab')}_2$  and Fab' was purified from 10 liters of culture supernatant. The culture supernatants were first concentrated by a DC10 concentrator (Amicon) using a S10Y30 cartridge (Amicon), washed with 20 liters of distilled water, reconstituted, and then washed with 10 mM sodium phosphate buffer at pH 8.0, and concentrated again. The concentrate was then loaded on a DE52 (Whatman) column pre-equilibrated with 10 mM sodium phosphate buffer at pH 8.0. The flow-through from the DEAE column was collected and adjusted to a pH of 6.8 and a conductivity of 1.0 ms/cm. The sample was next loaded onto a CM52 column, equilibrated with 10 mM sodium phosphate at pH 6.8. The CM52 column was eluted with 25 mM NaCl in 10 mM  $\text{NaPO}_4$ , pH 6.8. The eluant was collected and diluted with water to a conductivity of 1.0 ms/cm and loaded onto a second CM52 column equilibrated with 10 mM sodium phosphate at pH 6.8 and the bound antibody was eluted with a linear salt gradient from 0 to 25 mM NaCl in 10 mM sodium phosphate, pH 6.8. The fractions were analyzed by ELISA and SDS-PAGE and those that contained a mixture of  $\text{F(ab')}_2$  and Fab' were pooled and concentrated. The  $\text{F(ab')}_2$  and Fab' were purified away from each other using a TSK-125 gel filtration HPLC column. The purified Fab', and  $\text{F(ab')}_2$  ran at  $\sim 48$  Kd and  $\sim 100$  kd, respectively, in non-reducing SDS polyacrylamide gels. Samples from both the  $\text{F(ab')}_2$  and Fab' fractions resolved into light chain and Fd chain bands on reducing SDS polyacrylamide gels. During the purifications, it was observed that those

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constructs which contained two hinge cysteines carboxy to the light chain-Fd disulfide bond (pING1698, pING1699, and pING1800) had a lower level of heterogeneous forms than the construct with the single cysteine (pING1697), as evidenced by protein bands at other than ~48 kd and ~100 kd on non-reducing SDS polyacrylamide gels.

F(ab')<sub>2</sub> was generated by pepsin digestion of ING-4 IgG as follows. 25 ul of 50% slurry of immobilized pepsin (Pierce) was equilibrated with 400 µl of digestion buffer (20 mM sodium acetate, pH 4.5), and centrifuged at 1000 x g, 5 min. The immobilized pepsin was resuspended in 50 µl of digestion buffer. 50 µl of ING-4 (1 mg) was added to the pepsin suspension and incubated 4 hours, 30°C on a shaker. The digested IgG was extracted by adding 150 µl of 10 mM Tris, pH 7.5 followed by centrifugation at 1000 x g, 5 min. The supernatant, containing F(ab')<sub>2</sub>, was separated from undigested IgG and Fc fragment by passage through a 1 ml protein A-sepharose column. SDS-PAGE analysis revealed the presence of F(ab')<sub>2</sub> plus two lower molecular weight bands. F(ab')<sub>2</sub> was further purified on a TSK-125 HPLC column.

e. Binding characteristics of F(ab')<sub>2</sub> and Fab' secreted by yeast

The purification from yeast culture supernatant of proteins of the expected size of F(ab')<sub>2</sub> and Fab' suggests that yeast secrete correctly associated molecules. Direct and competition binding assays were used to confirm that these molecules are functional in binding to antigen. In direct binding assays, the various chimeric ING-4 F(ab')<sub>2</sub> and Fab' molecules bound to the same target cell as the chimeric ING-4 IgG and Fab (data not shown). In a competitive binding assay using HT29 cells and biotinylated ING-4 IgG, the yeast-produced pING1800 F(ab')<sub>2</sub> protein containing two interchain cysteines was equivalent to the chimeric ING-4 IgG (Figure 5). The F(ab')<sub>2</sub> proteins from pING1698 and

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pING1699 were also tested; their competitions of biotinylated ING-4 binding were also equivalent to ING-4 IgG (data not shown). By contrast, the competition of F(ab')<sub>2</sub> prepared by pepsin digestion of ING-4 was equivalent to ING-4 Fab, rather than to IgG (Figure 5), suggesting that pepsin digestion adversely affected the binding characteristics of the F(ab')<sub>2</sub>. Monovalent Fab' proteins containing either one (pING1697) or two (pING1698, pING1699, pING1800) Fd' interchain cysteines competed in a manner similar to that of monovalent ING-4 Fab (data not shown).

Example 3: Secretion of Functional H65 F(ab')<sub>2</sub> and Fab' by *Escherichia coli*

Bacteria such as *E. coli* are capable of secreting functional mouse-human chimeric Fab (Better *et al.*, *supra*). In the present example, *E. coli* serves as the host for the production of mouse-human chimeric F(ab')<sub>2</sub> and Fab' molecules containing Fd' modules with either one or two inter-heavy chain Fd' cysteines in the hinge domain carboxy to the cysteine that normally forms the light chain-heavy chain disulfide bond (*see* Figure 1b). The gene modules used for these experiments include Fd' module C (Figure 1b) encoding two inter-heavy chain cysteines plus nine additional amino acids on the carboxyl side of the last cysteine and several different Fd' modules A, E, and F (Figure 1b), with one inter-heavy chain cysteine. The chimeric antibody fragments in this example contain the light and heavy chain variable regions of a monoclonal antibody, designated as H65, which binds to the CD5 antigen on human T cells (Kernan *et al.* *J. Immunology* 133: 137-146 (1984)).

a. Dicistronic expression system for light chain and Fd' genes

Secretion of F(ab')<sub>2</sub> or Fab' by bacteria requires the simultaneous expression of the genes encoding both the light chain and Fd' chain each

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fused to a bacterial ribosome binding site and signal sequence. This is optimally achieved by fusing both genes to each other and placing the dicistronic operon under the control of a strong, inducible promoter. This example describes a system using the *pelB* signal sequence from *Erwinia carotovora* (Lei *et al. J. Bacteriol.* 169: 4379-4383 (1987)) and the *araBAD* promoter from *Salmonella typhimurium* (Horwitz *et al. Gene* 14: 309-319 (1981)) to produce chimeric H65 F(ab')<sub>2</sub> and Fab' in *E. coli*.

For production of H65 F(ab')<sub>2</sub> and Fab' containing Fd' modules A, C, E, and F (see Figure 1), the expression vectors pING3217, pING3219, pING3518, and pING3519 were constructed as shown in Figure 6 for pING3217.

To provide the variable-region sequences, H65 hybridoma cells secreting a mouse IgG1, kappa (Kernan *et al., supra*) were used for RNA isolation and cDNA preparation. After determination of heavy chain and light chain sequences, oligonucleotides were designed, synthesized, and used to prime the amplification of the VH-JH1 and VL-Jk1 coding sequences by polymerase chain reaction (PCR) using standard methods (PCR Protocols, Innis, ed., Academic Press, (1990)). The PCR-amplified V gene modules were ligated to *SacI*-digested plasmid pUC18 to generate pXOM1 (VH) and pXOM2 (VL). Figures 8 and 9 show the DNA sequences of pXOM1 and pXOM2. Subsequently, new primers were designed, synthesized and used to amplify the V region sequences encoding the fully processed VH and VL domains. The PCR primers were designed so that a blunt end would be present at the 5' end to join to a *pelB* leader peptide-encoding sequence prepared by treatment with *SstI*-restriction endonuclease and T4 polymerase to generate a blunt end at its 3' end. The PCR primers were also designed to include a *BstEII* site (JH1) or a *HindIII* site (Jk1) at the 3' end of the V region to match those of the pIT106 expression vector of Better *et al., supra*, and Robinson *et al.*, PCT US8802514. The primers used for VH PCR amplification are

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H65G1 (5'-AAC ATC CAG TTG GTG CAG TCT G-3') and H65G2 (5'-GAG GAG ACG GTG ACC GTG GT-3'), and for VL amplification were H65K1 (5'-GAC ATC AAG ATG ACC CAG T-3') and JK1-HindIII (5'-GTT TGA TTT CAA GCT TGG TGC-3').

The Fd' gene module A from Example 1 was used to assemble first pX15F, then pING3217 (Figure 6). Fd' gene module C was used in the same way to construct pING3219. For pING3518 and pING3519, Fd' gene modules E and F were generated by PCR gene amplification using Fd' gene modules A and C as templates. Synthetic oligonucleotides to introduce the desired sequence changes were synthesized and used in a PCR reaction to generate *SalI* to *XhoI* Fd' gene fragments for ligation to DNA fragments from the chimeric Fd' vector pX15F. The remainder of the assembly is the same as for pING3217 (Figure 6).

Descriptions of other starting plasmids in Figure 6 are:

1. pING1500 is identical to pRR187 described in Figure 38 of Robinson *et al.*, PCT US8802514, except for the insertion of a *XhoI* linker oligonucleotide into the unique *SalI* site.
2. pING3215 is identical to pING1500 except that it has the Fd' gene module A (Example 1).
3. pING3104 is described in Figure 13 of Better *et al.*, PCT US8903852.
4. pS2D contains the human Ck *HindIII* to *XhoI* sequence present in pING1431 described in Figure 27 of Robinson *et al.*, PCT US8802514.
5. pLE10 contains the human CH1 *BstEII* to *BstEII* fragment of pIT106 described by Better *et al.*, *supra*.

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b. Production of chimeric H65 F(ab')<sub>2</sub> and Fab' in bacteria

The plasmids pING3217, pING3219, pING3518, and pING3519 were transformed into *E. coli* strain E104. These strains were cultured in a Chemap 14-liter fermenter in 10 liters of minimal medium supplemented with 0.7% glycerol as a carbon source and induced by adding arabinose gradually over a period of 16 hours to a final concentration of 0.2% when an A<sub>600</sub>=100 was attained. The cells were incubated for 32 hours following the initiation of induction.

F(ab')<sub>2</sub> and Fab' were purified from 10 liters of culture supernatant using a process similar to that described for the yeast Fab'. The fermentation broth was passed through a 0.2 μM filter and concentrated five-fold by ultrafiltration (Amicon YM10 membrane). The media was replaced with 10 mM sodium phosphate buffer, pH 6.8, by diafiltration. After passing through a second 0.1 μM membrane, the concentrate was bound to a CM cellulose column and eluted with 0.10M sodium chloride in pH 6.8 sodium phosphate buffer. The eluate is concentrated by ultrafiltration, and F(ab')<sub>2</sub> and Fab' fragments are separated by size exclusion chromatography. The concentrated eluate containing no more than 500 mg total protein is loaded onto a 3.2 cm diameter x 120 cm high Sephacryl-S200® column preequilibrated in pH 7.4 sodium phosphate buffer. The sample volume is less than 2.5% of the total column volume. The resulting protein fractions were analyzed on a non-reducing SDS polyacrylamide gel stained with coomassie blue. The Fab' fractions containing the two selectively-reducible cysteine/Fd' module C (pING3219) resolved into the expected Fab' band plus small-molecular-weight proteolytic digestion products of the light and Fd chains (Figure 7, lanes 5 and 6). The Fab' band comprised greater than 50% of the relative area within these lanes as measured by densitometry scans of the coomassie blue-stained gel (see Table 1 below) and could be readily

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separated from the small-molecular-weight light and Fd' chain fragments by standard chromatographic methods.

The various Fab' proteins containing the single selectively reducible cysteine Fd' modules A, E, and F (pING3217, pING3518, and pING3519) also resolved into the expected Fab' band and the proteolytic digestion products of unassociated light and Fd chain bands (Figure 7). Depending on the isolate, the Fab' bands comprised 30-40% of the relative area within each lane as determined by densitometry scans of the Coomassie blue-stained gel (Table 1).

Table 1 SDS PAGE/Densitometry Analysis of Purified Bacterial Fab'					
Percent of Lane Total(b)					
Plasmid	Lane	Peak 1	Peak 2	Peak 3	Peak 4
pING3217	2	ND(a)	ND	ND	ND
pING3518	3	15	41	21	22
pING3519	4	28	31	20	22
pING3219	5	9	57	8	25
pING3219	6	1	56	3	35
(a) ND = not done					
(b) Values are reported as the percent of the total densitometer scan of each lane of the SDS gel shown in Figure 7.					

Surprisingly, these preparations contained three additional bands not observed with the Fab' containing the two cysteines (Figure 7, lanes 2, 3 and 4). Densitometry scans of these lanes of the gel established that the additional bands comprised approximately 20% of the relative area (Table 1). N-terminal amino acid analysis established that the largest of these bands consisted of light chain, while the other two consisted of two

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different forms of processed Fd chain. The purification of Fab' protein was unusual and problematic because intact Fab' could only be separated from these additional non-disulfide-bonded forms by treatment with guanidine hydrochloride followed by urea exchange prior to separation by ion exchange chromatography. Further experiments established that only the light chain component of the three bands contained a selectively-reducible thiol available for conjugation with RTA-30, suggesting that the Fd' bands contained no selectively-reducible thiol groups. These results demonstrate that, unexpectedly, human IgG1 Fab' constructs with a single hinge cysteine carboxy to the cysteine normally bonded to light chain result in the production of a mixture of non-covalently associated Fab' as well as Fab' with correct disulfide bonds.

The F(ab')<sub>2</sub> fractions gave the expected higher molecular weight band, indicating the presence of properly formed, bivalent F(ab')<sub>2</sub>. As found in Example 1, there was a higher yield and less heterogeneity observed for F(ab')<sub>2</sub> from the two cysteine pING3219 construct than from any of the single cysteine constructs.

c. Binding characteristics of H65 Fab' and F(ab')<sub>2</sub> produced by *E. coli*

The bacterially-produced Fab' containing the single selectively-reducible hinge cysteine (pING3217) was assessed for function in a competitive binding assay using Molt-4 cells and FITC-labeled H65 IgG. In this assay, CD5+ Molt 4 cells (10<sup>6</sup> ml) were mixed with varying concentrations (1 to 4 µg/ml) of the H65 IgG or Fab', together with a fixed concentration (0.2 µg/ml) of H65-FITC. Following a 1-hour incubation at 4°C in the dark, cell-associated H65-FITC was measured by flow cytometry. Binding of the Fab' fragments relative to H65 IgG was then quantitated by comparing the slopes of the competitive binding curves for each sample. Intact H65 IgG would therefore give a value of

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100% in this assay. On a weight basis, H65 Fab' exhibited a binding affinity equivalent to that of the parental murine H65 IgG. On a molar basis, this value is roughly 30% that of the parental IgG. Identical results were obtained with the Fab' containing two selectively reducible hinge cysteines. In separate experiments, *E. coli*-produced H65 F(ab')<sub>2</sub> was found to have approximately 80% of the binding affinity of the parental murine H65 IgG, as measured on a molar basis.

Example 4: Secretion of Functional 4A2 Fab' by *E. coli*

Bacterial expression vectors for 4A2 Fab' were constructed in a similar manner to those for H65 Fab' (Example 3). In this example, the mouse hybridoma cell line producing the 4A2 antibody served as the starting point for isolation of the antibody genes.

a. Construction of expression vectors

One liter of hybridoma cells which produce antibody 4A2 were collected and polyA RNA was prepared. A cDNA library was prepared from this RNA, and individual cDNA clones containing the full length light chain (p4A2K-13) and heavy chain (p4A2g-12) were identified by hybridization to mouse constant region probes. The DNA sequence of each variable region was determined and is shown in Figures 10 and 11. The junction region of the 4A2 light chain (kappa) was JK1 and that of the heavy chain was JH3. Specific primers were used to PCR amplify the variable and junction (V-J) segments from each of the genes from p4A2K-13 and p4A2g-12 for subsequent cloning into expression vectors. The N-terminal amino acid sequence for both the light and heavy chain proteins was also determined to assure that the correct genes were identified and

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used for subsequent cloning steps. For cloning into bacterial expression vectors, PCR amplification of the light chain V-J region was with primers:

5'-GACATTGTGCTCACCCAATC-3' and

5'-GTTTGATTTC AAGCTTGGTGC-3',

while the heavy chain V-J region was amplified with:

5'-GAAGTGCAGCTGGTGGAGTC-3' and

5'-GAGACGGTGACCAGAGTCCCT-3'.

The DNA fragment containing the light chain V-J region was digested with *Hind*III and cloned as a blunt to *Hind*III fragment into pING1500 along with the human kappa constant region to generate pZ1G. Similarly, the DNA fragment containing the heavy chain V-J region was digested with *Bst*EII and cloned as a blunt to *Bst*EII fragment into pING3215 along with a *Bst*EII fragment from CH1 to generate pD28H. Plasmids pZ1G and pD28H were used to assemble the 4A2 Fab' expression vector, pING3218. This cloning scheme is outlined in Figure 12.

Plasmid pING3218 contains Kappa and Fd', where the C-terminus of Fd' is as shown in Figure 1 gene module A. A derivative of this plasmid, pING3197, was constructed that has an Fd' C-terminus as shown in Figure 1, gene module C. Plasmid pING3197 was derived from pZ1G and pD28H where the genes were assembled in the order kappa followed by Fd (opposite order to pING3218), and the Fd' sequence illustrated on Figure 1 gene module C was introduced as a *Sau*I to *Xho*I fragment from pING3219 (Example 3).

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b. Production of chimeric 4A2 F(ab')<sub>2</sub> and Fab' in bacteria

Plasmids pING3218 and pING3197 were transformed into *E. coli* strain E104. These strains were cultured in a Chemap 14-liter fermenter in 10 liters of minimal medium supplemented with 0.7% glycerol as a carbon source and induced by adding arabinose to 0.05% when an A<sub>600</sub>=50 was attained. The cells were incubated for 24 hours following induction.

F(ab')<sub>2</sub> and Fab' from either pING3218 or pING3197 were purified from 10 liters of culture supernatant using a process similar to that described for the H65 F(ab')<sub>2</sub> and Fab' from either yeast or *E. coli*. The fermentation broth was passed through a 0.1 μM filter and concentrated by ultrafiltration (Amicon S10Y10 membrane) and adjusted to pH 6.5. This concentrate was loaded onto a CM52 column at pH 6.5 in 10 mM phosphate buffer. The CM52 column was eluted with 0.02 N NaCl, and concentrated to 50 ml. SDS-PAGE and Western blot analysis of the concentrate from pING3218 (two hinge cysteines) fermentations showed three major bands containing light chain: F(ab')<sub>2</sub>, Fab', and free light chain. However, pING3197 (1 hinge cysteine) gave about 7 major bands, indicating a higher degree of heterogeneity of products than obtained from pING3218. This material was loaded in batches to a CBX Prep15 HPLC column and eluted with a 0 to 0.15 N NaCl gradient in 10 mM phosphate pH 6.5. The purified F(ab')<sub>2</sub> and Fab' are prepared by pooling appropriate fractions, as assessed by SDS-PAGE.

Example 5: Establishment of Conditions for Reduction of F(ab')<sub>2</sub>

The Fab' molecules described in Examples 2, 3, and 4 should contain cysteine thiol groups which can be used for *in vitro* coupling to a variety of molecules. Initial attempts to couple two Fab' molecules to

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form a  $F(ab')_2$  were unsuccessful, suggesting that the cysteine thiol groups on these molecules were blocked, possibly due to adduct formation. This hypothesis was confirmed by testing with Ellman's reagent.

In the present example, we establish conditions for the selective reduction of the Fd cysteine residue(s) on the carboxyl side of the Fd-light chain disulfide bond. Yeast-derived chimeric ING-4  $F(ab')_2$  containing two inter-heavy chain cysteines plus nine additional amino acids (see Figure 1(b)(C) and *E. coli*-derived chimeric H65 Fab' containing a single hinge chain cysteine carboxy to the Fd-light chain disulfide-linked cysteine were used to establish the reduction conditions.

ING-4  $F(ab')_2$  produced by yeast and purified as described in Example 2 was incubated for 4 hours at 4°C in 20 mM Tris-HCl, pH 8.0 containing dithiothreitol (DTT) at concentrations from 0.05 to 1 mM. The proteins subsequently were analyzed by SDS-PAGE, followed by coomassie blue staining. A DTT concentration of 0.5 mM was sufficient to reduce the  $F(ab')_2$  molecules to Fab' without affecting the Fd-light chain disulfide bond. DTT concentrations of 1 mM or higher reduced the  $F(ab')_2$  molecules to individual Fd and light chains, as assessed by SDS-PAGE.

The H65 Fab' produced by *E. coli* was tested as above and also required 0.5 mM DTT in 20 mM Tris-HCl, pH 8.0 to selectively reduce the single hinge cysteine nearest the carboxy terminus of Fd without affecting the Fd-light chain disulfide bond. Following removal of excess DTT by gel filtration in 0.1N acetic acid, Ellman's reagent was used to establish that there were approximately 1.4 thiol groups per Fab' molecule.

Example 6: Formation of F(ab')<sub>2</sub> from Fab'

Fab' molecules with selectively-reducible thiol groups can be used to form either homodimeric or bifunctional heterodimeric F(ab')<sub>2</sub> molecules. Homodimeric Fab' molecules should retain the affinity of an IgG molecule. Heterobifunctional F(ab')<sub>2</sub> molecules could include specificities toward two different cell types or a cell and a ligand.

In the present example, chimeric ING-2 and ING-4 Fab' molecules containing Fd chains with one or two cysteines at the C terminal side of the Fd-light chain disulfide bond are used for the production of homodimeric and bifunctional, heterodimeric F(ab')<sub>2</sub> molecules.

I. Production of Homodimeric F(ab')<sub>2</sub> Molecules

a. Formation of F(ab')<sub>2</sub> from Fab'

ING-4 Fab' molecules containing an Fd chain with either one or two cysteines on the carboxyl side of the Fd-light chain disulfide bond (*see* Example 1) were used to form F(ab')<sub>2</sub>. Adducts associated with these additional Fd cysteines were removed by incubating 50 µg of the various Fab' proteins in 20 mM Tris-HCl, pH 8.0, containing dithiothreitol at 0.5 mM DTT in a volume of 50 µl for 4 hours at 4°C. The reduced Fab' proteins were then diluted to 20 µg/ml into a cold (4°C) aqueous solution comprised of 2 to 5 mM cysteine in 50 mM Tris-HCl, pH 7.8, and incubated overnight at 4°C to allow reassociation to occur. The solutions were then concentrated by Centricon® 10 to a volume of approximately 50 µl and F(ab')<sub>2</sub> formation was quantitated by densitometric scanning of coomassie blue-stained bands following SDS-PAGE. For further characterization, the F(ab')<sub>2</sub> can be separated from Fab' by gel filtration.

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Only the Fab' molecules containing two selectively-reducible hinge cysteines carboxy to the interchain disulfide cysteine formed F(ab')<sub>2</sub> molecules using the above conditions. Of the three Fab' constructs with two cysteines (*see* Example 1), the one with a nine amino acid tail appeared to give the best conversion to F(ab')<sub>2</sub>. The failure to produce F(ab')<sub>2</sub> using the established conditions with the Fab' containing one selectively-reducible cysteine is explained by an observation that H65 F(ab')<sub>2</sub> with one cysteine produced directly by *E. coli* could be reduced to Fab' by treatment with the same concentration of cysteine (2 mM) as used in the reassociation buffer. Thus, 2 mM cysteine maintains a reduced state for Fab' containing a single selectively-reducible cysteine but not for Fab' with two selectively-reducible cysteines.

b. Binding characteristics of *in vitro*-formed F(ab')<sub>2</sub>

The *in vitro* production of proteins of the expected size for F(ab')<sub>2</sub> suggests that the reassociation procedure resulted in the formation of correctly associated molecules. Competition binding assays were used to confirm that these molecules are functional. In a competition binding assay using HT29 cells and biotinylated ING-4 IgG, the various *in vitro*-produced F(ab')<sub>2</sub> proteins containing two inter-heavy chain cysteines competed equivalently to the chimeric ING-4 IgG and F(ab')<sub>2</sub> produced by direct secretion from yeast (data not shown).

## II. Production of Heterobifunctional $F(ab')_2$ Molecules

### a. Formation of $F(ab')_2$ molecules from Fab'

Heterobifunctional  $F(ab')_2$  molecules can be made by reducing the Fab' of two different specificities, either alone or in equimolar mixture, followed by simultaneous addition of the reduced Fab' molecules to the reassociation buffer described above. Using this procedure, a heterobifunctional  $F(ab')_2$  was constructed with chimeric ING-2 and ING-4 Fab' molecules. Chimeric ING-2 Fab is described by Better *et al.*, PCT US8903852; and chimeric ING-2 Fab' was made in the same manner as described above for ING-4 Fab'. Analysis of the reassociated protein on non-reducing, Coomassie blue-stained SDS gels demonstrated the presence of  $F(ab')_2$  protein of the expected molecular weight.

### b. Characteristics of heterobifunctional $F(ab')_2$ molecules

The heterobifunctional nature of this construct was confirmed by tests which make use of the fact that the ING-2 antibody contains a lambda light chain while the ING-4 antibody contains a kappa light chain. A sandwich ELISA using antisera against human lambda as the coat and against human kappa as the second antibody revealed the presence of  $F(ab')_2$  with both kappa and lambda light chains. Direct binding assays can be performed with the  $F(ab')_2$  using HT29 and BT20 cells or membrane preparations derived from these cells, which are specifically recognized by ING-4 (kappa light chain) and ING-2 (lambda light chain), respectively.

Example 7: Construction and Activity of Fab'-RTA30 Conjugates

Immunotoxins (antibody-toxin conjugates) are frequently prepared by randomly derivatizing an antibody with the heterobifunctional crosslinker SPDP, followed by a disulfide-exchange reaction with the free thiol of ricin toxin A chain (RTA). The disulfide linkage so produced is essential for maximal conjugate cytotoxic activity (Blakey *et al. Prog. Allergy* 115: 50 (1988)). Since the microbially-produced Fab' fragments described in the present invention contain a selectively-reducible thiol on the Fd chain, the process of disulfide conjugation is greatly simplified and highly specific.

Similarly, the procedures outlined below could be used to generate conjugates between a Fab' and any other thiol-containing toxin (*viz.*, abrin A chain), or to create monospecific or bispecific F(ab')<sub>2</sub> fragments linked by a disulfide bridge. In conjunction with appropriate linkers, such as SPDP or 2-iminothiolane, conjugates could also be prepared between Fab' fragments and compounds that do not contain selectively-reducible thiols, such as the type I ribosome-inactivating proteins (gelonin, saporin, pokeweed antiviral protein, the barley RIP, etc.), enzymes (alkaline phosphatase), or drugs (daunomycin, etc.).

The number of RTA30 molecules conjugated to a Fab' is dependent on the reaction conditions and the number of selectively reducible thiols on the Fab'. For example, the Fab' from construct pING3219 has two selectively reducible thiols on the Fd chain, and was conjugated by the methods shown below to make an active Fab'-immunoconjugate with 2 RTA molecules per Fab'.

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## a. Preparation of H65 Fab'-RTA30

In this example, the H65 Fab' containing the single selectively-reducible hinge cysteine described in Example 3 and the aromatic disulfide dithiobis(pyridine-N-oxide) were used to prepare a disulfide-crosslinked conjugate with the 30 kD form of RTA (RTA30). H65 Fab' (86 mg, 2.4 mg/ml in PBS, pH 7.0) was reduced by the addition of dithiothreitol to a final concentration of 2 mM with rapid stirring. Following incubation for 1 hour at 25°C, dithiobis(pyridine-N-oxide) was added to 7 mM and incubation was continued for 1 additional hour. This process both deblocks the Fab' thiol and activates this thiol in preparation for conjugation. The thiol-activated H65 Fab' was then recovered by desalting on a 5 x 30 cm column of Trisacryl® GF-05LS equilibrated in PBS, pH 7.0. The number of activated thiol groups per Fab' was found to be 1.05, as measured spectrophotometrically following the addition of Ellman's reagent.

Prior to conjugation, RTA30 (300 mg, 6 mg/ml in PBS) was reduced by adding DTT to 50 mM for 30 minutes at 25°C, divided in half, and each half was desalted on a 5 x 30 cm column of Trisacryl GF-05LS equilibrated in PBS. The RTA30-SH contained 0.91 SH/mol, and was concentrated by ultrafiltration on an Amicon YM10® membrane to 6 mg/ml. The thiol-activated H65 Fab' (82 mg) was then mixed with a five-fold molar excess (246 mg) of the freshly-reduced RTA30 and incubated at 25°C for 3 hours. The final concentrations of activated Fab' and RTA30 were 1.0 and 3.0 mg/ml, respectively.

Following an additional incubation for 15 hours at 4°C, the Fab'-RTA30 conjugate was purified from the reaction mixture by sequential affinity chromatography on protein G and Cibachron Blue® F3GA resins. First, residual-free RTA30 was removed by applying the mixture to a 10 ml column of GammaBind Plus® (Genex) previously equilibrated in PBS.

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The column was washed with PBS until the absorbance at 280 nm approached zero; Fab'-RTA30 and any remaining unconjugated Fab' were eluted with 0.5 M ammonium acetate, pH 3.0. This material was immediately neutralized with saturated Trizma® base and dialyzed against 10 mM Tris-HCl, 150 mM NaCl, pH 8.0. Next, the small amount of contaminating free Fab' was removed by diluting the dialyzed protein G eluate 1:1 with 10 mM Tris-HCl, pH 8.0, and then applying the mixture to a 10 ml column of Blue ToyoPearl® (TosoHaas) equilibrated in 10 mM Tris-HCl, 75 mM NaCl, pH 8.0. After washing the column with equilibration buffer, purified H65 Fab'-RTA30 was batch eluted with 1 M NaCl in equilibration buffer. The final conjugate (65.4 mg) exhibited only a single, symmetrical peak when analyzed by size-exclusion HPLC, and contained one RTA30/Fab'.

b. Evaluation of H65 Fab'-RTA30

The antigen binding and cytotoxic properties of the H65 Fab'-RTA30 conjugate were evaluated in several systems. Antigen reactivity was assessed by using the competitive binding assay described in Example 3(c). Relative to unconjugated H65 Fab', binding of the Fab-RTA30 conjugate was virtually 100% (30% vs. 27% of control on a molar basis, respectively). Although these values are lower (roughly 1/3 on a molar basis) than that obtained with the parental, bireactive H65 antibody, these results indicate that the process of conjugation has little effect on the reactivity with antigen.

The cytotoxicity of the H65 Fab'-RTA30 conjugate was examined on several different cell types. Figure 13 shows the results of a representative experiment in which cells from the CD5 T-cell line HSB2 ( $5 \times 10^5$  per ml) were incubated with increasing concentrations of conjugate for up to 24 hours, at which time the ability of the cells to

synthesize protein was quantitated by measuring the incorporation of  $^3\text{H}$ -leucine. On a weight basis, the Fab'-RTA30 conjugate inhibited protein synthesis in these cells by 50% (the IC<sub>50</sub>) at a concentration of 43 ng/ml. Based upon a molecular weight of 80,000 daltons, this translates to an IC<sub>50</sub> of 0.5 nM. The curves obtained for the free H65 Fab', as well as for the intact IgG H65-RTA30 conjugate are also shown for comparison.

H65 Fab'-RTA30 was also tested against human peripheral blood mononuclear cells (PBMC) by an assay which measures the inhibition of DNA synthesis as an index of cellular cytotoxicity. PBMC obtained from normal healthy donors were isolated over Ficoll-Hypaque and washed extensively. Phytohemagglutinin (PHA) blasts were obtained by activating PBMC in bulk culture for 3 days with an optimal concentration of PHA. Both cell populations were incubated with samples for a total of 90 hours;  $^3\text{H}$ -thymidine was added 16 hours prior to cell harvest.

The cytotoxicity of H65 Fab'-RTA30 for resting and PHA-activated PBMCs is shown in Figure 14. In these assays, both H65 Fab'-RTA30 and the control H65 IgG-RTA30 exhibited similar IC<sub>50</sub> values, which averaged ca. 100 ng/ml for each of the two cell populations. Control IgG (IND1-RTA30) or Fab' (ING-2RTA30) conjugates were nontoxic at the concentrations tested. On a molar basis, the H65 Fab'-RTA30 conjugate was thus roughly 1/3 as active as the IgG conjugate. Interestingly, under both sets of experimental conditions (resting and activated PBMCs), the H65 Fab'-RTA30 conjugate exhibited a greater degree of cell killing than did the corresponding H65 IgG-RTA30 conjugate. To a lesser extent, this trend to more complete killing with the Fab'-RTA30 conjugate was also noted with each of the cell lines tested (data not shown).

These results demonstrate that immunotoxin conjugates derived from microbially-produced Fab' molecules can be similar in potency to immunotoxins derived from animal cell-produced whole antibodies. The H65-RTA control whole antibody immunotoxin in Figure 8 has been

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shown to be useful in the therapy of steroid-resistant graft vs. host disease (Kernan *et al.*, *J. Amer. Med. Assoc.* 259: 3154-3156 (1988)). Thus, the H65 Fab-RTA in this example and similar T-cell-reactive molecules of this invention may be therapeutically useful in the treatment of T lymphocyte diseases and disorders.

#### Example 8: Construction and Activity of F(ab')<sub>2</sub>-RTA30 Conjugates

As described in the previous example, immunotoxins are typically composed of an antibody linked to a ribosome-inactivating protein (such as RTA) via a reducible disulfide bond. In this format advantage is taken of the intrinsic bivalency of the intact antibody molecule, thereby providing a targeting moiety with an affinity for target cells generally higher than is typical for monovalent antibody fragments. The F(ab')<sub>2</sub> fragments described in the present invention offer an alternative to intact antibodies for the preparation of immunotoxins. In addition to providing high affinity binding, F(ab')<sub>2</sub> fragments lack Fc receptors which may cause non-specific uptake by macrophages and other cells of the immune system. Although the present example employs an F(ab')<sub>2</sub> molecule composed of Fab' fragments with identical antigen specificity, similar methodologies could be used to create bivalent immunotoxins with heterologous Fab' fragments. Such conjugates could be targeted to different cell-surface antigens, or to different epitopes on the same antigen. Moreover, non-disulfide linked conjugates to F(ab')<sub>2</sub> fragments could also be prepared by appropriate linker selection.

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a. Preparation of H65 F(ab')<sub>2</sub>-RTA30

In order to prepare the desired conjugate, the F(ab')<sub>2</sub> fragments must first be derivatized with a crosslinking reagent so as to introduce a reactive thiol group necessary for conjugation. In this example, the H65 F(ab')<sub>2</sub> molecule described in Example 3 was first reacted with the heterobifunctional crosslinking reagent 5-methyl-2-iminothiolane (M2IT; (see Goff *et al.*, *Bioconjugate Chem.* 1: 381-386 (1990); and U.S. Patent No. 4,970,303) and the aromatic disulfide dithionitrobenzoic acid (DTNB). This reaction both introduces a free SH group via the M2IT linker, and then activates this thiol with a thionitrobenzoic acid (TNB) leaving group in preparation for the disulfide-exchange reaction with RTA30-SH.

H65 F(ab')<sub>2</sub> (65 mg: 2.9 mg/ml in 25 mM triethanolamine, 150 mM NaCl, 2.5 mM DTNB, pH 8.0) was reacted with a 12-fold molar excess of M2IT at 25°C. Under these conditions, the F(ab')<sub>2</sub> fragments are first derivatized with M2IT, and then the newly exposed linker thiol becomes activated with TNB. After 70 min with constant stirring, the thiol-activated H65 F(ab')<sub>2</sub>-(M2IT)-TNB was recovered by desalting on a 2.5 cm x 40 cm column of Trisacryl® GF05LS equilibrated in 0.1 M NaPO<sub>4</sub>, 0.1 M NaCl, pH 7.5, at 4°C. Spectrophotometric analysis of DTT-treated samples indicated that 1.9 activated thiols had been introduced into the H65 F(ab')<sub>2</sub> fragments.

For conjugation, the thiol-activated H65 F(ab')<sub>2</sub>-(M2IT)-TNB (62 mg) was mixed with a three-fold molar excess (58 mg) of RTA30-SH (prepared as described in Example 7) and the mixture was allowed to incubate at 25°C for 2 hr. The final concentrations of F(ab')<sub>2</sub> and RTA30 were 1.4 and 1.3 mg/ml, respectively. Following an additional 16 hr at 4°C, the H65 F(ab')<sub>2</sub>-(M2IT)-RTA30 conjugate was purified away from residual unreacted F(ab')<sub>2</sub> fragments and RTA30 by sequential affinity chromatography on Protein G® and Cibacron Blue® F3GA resins

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as described in the preceding example. The final conjugate (15 mg) contained a mixture of  $F(ab')_2$  fragments containing 1 or 2 RTA30 molecules, with an average RTA  $F(ab')_2$  ratio of 1.3.

b. Evaluation of H65  $F(ab')_2$ -RTA30

Antigen reactivity of the H65  $F(ab')_2$ -(M2IT)-RTA30 conjugate was examined in the competitive binding assay as described in Example 3(c). Relative to the intact H65 antibody, binding of the  $F(ab')_2$ -RTA conjugate was actually greater (147%) on a molar basis. This result is similar to that seen for the intact H65 antibody conjugate (153% relative to antibody). Thus, the  $F(ab')_2$ -RTA conjugate binding was 97% that of the corresponding intact antibody conjugate.

Similarly, the cytotoxic activity of the  $F(ab')_2$  conjugate was examined against several cell types, as described in Example 7(b). Against HSB2 cells, the IC50 was 1.8 ng/ml (13 pM), relative to 23 ng/ml (112 pM) for the intact antibody H65-RTA conjugate. In addition, as was noted with the Fab'-RTA30 conjugate of Example 7, the  $F(ab')_2$  conjugate killed the target cells to a greater extent (92%) than did H65-RTA (78%). Against CEM cells, the IC50 of H65  $F(ab')_2$ -(M2IT)-RTA30 was 22 ng/ml (158 pM), vs. 43 ng/ml (214 pM) for H65-RTA. For PBMCs, the IC50 was 9.5 ng/ml (68 pM) and the extent of kill was 89%, relative to 26 ng/ml (124 pM) and 66% for H65-RTA. Thus, the RTA30 immunoconjugate of microbially-produced  $F(ab')_2$  shows a more potent and complete killing of a variety of target CD5 T cells than does the immunoconjugate of parental mouse antibody.

Having fully described the invention, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made hereto without departing from the spirit or scope of the invention as set forth herein.

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: MICROBIALY-PRODUCED ANTIBODY FRAGMENTS  
AND THEIR CONJUGATES

(iii) NUMBER OF SEQUENCES: 25

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(v) COMPUTER READABLE FORM:

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(B) COMPUTER: IBM PC compatible  
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(viii) ATTORNEY/AGENT INFORMATION:

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- 44 -

(B) REGISTRATION NUMBER: 29,021

(C) REFERENCE/DOCKET NUMBER: 0610.0830000

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## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro
1				5						10			

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

- 45 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ACATGCCCAC CA

12

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACATGCCCAC CATGCCCAGC T

21

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 42 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACATGCCCAC CATGCCCAGC TCCTGAATTG TTGGCTGGTC CA

42

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## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ACATGCCAC CGTGCCAGC ACCTGAACTC CTGGGGGGAC CG

42

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser  
1 5 10 15

Arg Thr Pro Asp  
20

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## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Thr	Ser	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro
1				5						10			

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ACATCTCCAC CATGC

15

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- 48 -

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ACATCTCCAC CATGCCAGC TCCTGAATTG TTGGCTGGTC CA

42

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTCCACCATG ATCAC

15

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TCGAGTGATC ATGGTGGAC

19

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTCCAGCTTG ATCACTCGAG G

21

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AATTCCTCGA GTGATCAAGC TGGAC

25

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro
1				5					10

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 42 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

- 51 -

GTCCAGCTCC TGAATTGTTG GGTGGTCCAT GATCACTCGA GG

42

## (2) INFORMATION FOR SEQ ID NO:16:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 46 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AATTCCTCGA GTGATCATGG ACCACCCAAC AATTCAGGAG CTGGAC

46

## (2) INFORMATION FOR SEQ ID NO:17:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ACCCCTGACT GATCA

15

## (2) INFORMATION FOR SEQ ID NO:18:

- 52 -

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 414 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

ATGGCTTGGG TGTGGACCTT GCTATTCCTG ATGGCAGCTG CCCAAAGTGC CCAAGCACAG	60
ATCCAGTTGG TGCAGTCTGG ACCTGAGCTG AAGAAGCCTG GAGAGACAGT CAAAATCTCC	120
TGCAAGGCTT CTGGGTATAC CTCACAAAC TATGGAATGA ACTGGGTGAA GCAGGCTCCA	180
GGAAAGGGTT TAAGGTGGAT GGGCTGGATA AACACCCACA CTGGAGAGCC AACATATGCT	240
GATGACTTCA AGGGACGGTT TGCCTTCTCT TTGGAAACGT CTGCCAGCAC TGCCTATTTA	300
CAGATCAACA ACCTCAAAAA TGAGGACACG GCTACATATT TCTGTACAAG ACGGGGTTAC	360
ACTGGTACT TCGATGTCTG GGGCGCAGGG ACCACGGTCA CCGTCTCCTC AGCC	414

## (2) INFORMATION FOR SEQ ID NO:19:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 138 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

- 53 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Met Ala Trp Val Trp Thr Leu Leu Phe Leu Met Ala Ala Ala Gln Ser  
 1 5 10 15

Ala Gln Ala Gln Ile Gln Leu Val Gln Ser Gly Pro Glu Leu Lys Lys  
 20 25 30

Pro Gly Glu Thr Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe  
 35 40 45

Thr Asn Tyr Gly Met Asn Trp Val Lys Gln Ala Pro Gly Lys Gly Leu  
 50 55 60

Arg Trp Met Gly Trp Ile Asn Thr His Thr Gly Glu Pro Thr Tyr Ala  
 65 70 75 80

Asp Asp Phe Lys Gly Arg Phe Ala Phe Ser Leu Glu Thr Ser Ala Ser  
 85 90 95

Thr Ala Tyr Leu Gln Ile Asn Asn Leu Lys Asn Glu Asp Thr Ala Thr  
 100 105 110

Tyr Phe Cys Thr Arg Arg Gly Tyr Asp Trp Tyr Phe Asp Val Trp Gly  
 115 120 125

Ala Gly Thr Thr Val Thr Val Ser Ser Ala  
 130 135

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 388 base pairs

- 54 -

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATGGACATGA GGACCCCTGC TCAGTTTCTT GGAATCTTGT TGCTCTGGTT TCCAGGTATC	60
AAATGTGACA TCAAGATGAC CCAGTCTCCA TCTTCCATGT ATGCATCTCT GGGAGAGAGA	120
GTCACATCA CTTGCAAGGC GAGTCAGGAC ATTAATAGCT ATTTAAGCTG GTTCCAGCAG	180
AAACCAGGGA AATCTCCTAA GACCCTGATC TATCGTGCAA ACAGATTGGT AGATGGGGTC	240
CCATCAAGGT TCAGTGGCAG TGGATCTGGG CAAGATTATT CACTCACCAT CAGCAGCCTG	300
GACTATGAAG ATATGGGAAT TTATTATTGT CAACAGTATG ATGAGTCTCC GTGGACGTTC	360
GGTGGAGGCA CCAAGCTGGA AATCAAAC	388

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 129 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

- 55 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Met Asp Met Arg Thr Pro Ala Gln Phe Leu Gly Ile Leu Leu Leu Trp  
1                      5                      10                      15

Phe Pro Gly Ile Lys Cys Asp Ile Lys Met Thr Gln Ser Pro Ser Ser  
20                      25                      30

Met Tyr Ala Ser Leu Gly Glu Arg Val Thr Ile Thr Cys Lys Ala Ser  
35                      40                      45

Gln Asp Ile Asn Ser Tyr Leu Ser Trp Phe Gln Gln Lys Pro Gly Lys  
50                      55                      60

Ser Pro Lys Thr Leu Ile Tyr Arg Ala Asn Arg Leu Val Asp Gly Val  
65                      70                      75                      80

Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Gln Asp Tyr Ser Leu Thr  
85                      90                      95

Ile Ser Ser Leu Asp Tyr Glu Asp Met Gly Ile Tyr Tyr Cys Gln Gln  
100                      105                      110

Tyr Asp Glu Ser Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile  
115                      120                      125

Lys

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 393 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

- 56 -

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATGGAGTCAG ACACACTCCT GCTATGGGTG CTGCTGCTCT GGGTTCCAGG CTCCACTGGT	60
GACATTGTGC TCACCCAATC TCCAGCTTCT TTGGCTGTGT CTCTGGGGCA GAGAGCCACC	120
ATCTCCTGCA GAGCCAGTGA AAGTGTGAA TATTATGGCA CAAGTTAAT GCAGTGGTAC	180
CAACAGAAAC CAGGACAGCC ACCCAAATC CTCATCTATG CTGCATCCAA CGTAGAATCT	240
GGGGTCCCTG CCAGGTTTAG TGGCAGTGGG TCTGGGACAG ACTTCAGCCT CAACATCCAT	300
CCTGTGGGGG AGGAGGATAT TGCATGTAT TTCTGTCAGC AGAGTAGGAA GGTCCTTGG	360
ACGTTCCGGT GAGGCACCAA GCTGGAAATC AAA	393

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 131 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

- 57 -

Met Glu Ser Asp Thr Leu Leu Leu Trp Val Leu Leu Leu Trp Val Pro  
 1 5 10 15

Gly Ser Thr Gly Asp Ile Val Leu Thr Gln Ser Pro Ala Ser Leu Ala  
 20 25 30

Val Ser Leu Gly Gln Arg Ala Thr Ile Ser Cys Arg Ala Ser Glu Ser  
 35 40 45

Val Glu Tyr Tyr Gly Thr Ser Leu Met Gln Trp Tyr Gln Gln Lys Pro  
 50 55 60

Gly Gln Pro Pro Lys Leu Leu Ile Tyr Ala Ala Ser Asn Val Glu Ser  
 65 70 75 80

Gly Val Pro Ala Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Ser  
 85 90 95

Leu Asn Ile His Pro Val Gly Glu Glu Asp Ile Ala Met Tyr Phe Cys  
 100 105 110

Gln Gln Ser Arg Lys Val Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu  
 115 120 125

Glu Ile Lys  
 130

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 426 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

- 58 -

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ATGAAC TTCG GGCTCAGCTT GATTTTCCTT GTCCTTGTTT TAAAAGGTGT CCAGTGTGAA	60
GTGCAGCTGG TGGAGTCTGG GGGAGGCTTA GTGAAGCCTG GAGGGTCCCT GAAACTCTCC	120
TGTGCAGCCT CTGGATTCAC TTTCAGTGAC TTTTACATGT ATTGGGTTCTG CCAGACTCCG	180
GAAAAGAGGC TGGAGTGGGT CGCAACCATT AGTGATGGTG GTATTTACAC CTACTATTCA	240
GACAGTGTGA TGGGGCGATT CACCATCTCC AGAGACAATG CCAAGAACAA CCTGTACCTG	300
CAAATAAGCA GTCTGAAGTC TGAGGACACA GCCATGTATT ACTGTGCAAG AGATCCCTAT	360
TCCTACGATA GTAGCCCCGC CTGGTTTGCT TACTGGGGCC AAGGGACTCT GGTCACGTGC	420
TCTGCA	426

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 142 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

- 59 -

Met Asn Phe Gly Leu Ser Leu Ile Phe Leu Val Leu Val Leu Lys Gly  
1                      5                      10                      15

Val Gln Cys Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Lys  
20                      25                      30

Pro Gly Gly Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe  
35                      40                      45

Ser Asp Phe Tyr Met Tyr Trp Val Arg Gln Thr Pro Glu Lys Arg Leu  
50                      55                      60

Glu Trp Val Ala Thr Ile Ser Asp Gly Gly Ile Tyr Thr Tyr Tyr Ser  
65                      70                      75                      80

Asp Ser Val Met Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn  
85                      90                      95

Asn Leu Tyr Leu Gln Ile Ser Ser Leu Lys Ser Glu Asp Thr Ala Met  
100                      105                      110

Tyr Tyr Cys Ala Arg Asp Pro Tyr Ser Tyr Asp Ser Ser Pro Ala Trp  
115                      120                      125

Phe Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ala  
130                      135                      140

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**WE CLAIM:**

1. A polynucleotide molecule comprising a bacterial promoter sequence operably linked to a sequence encoding the Fd polypeptide of an immunoglobulin Fab' or F(ab')<sub>2</sub> fragment molecule.
2. The molecule of claim 1 wherein the Fd sequence encodes two or more cysteine residues in the Fd hinge region on the carboxyl side of the disulfide bond formed between the light chain and the Fd chain.
3. The molecule of claim 2 wherein there are less than 30 amino acid residues between the cysteine residue with the highest amino acid number and the carboxyl terminal.
4. The molecule of claim 1 wherein the Fd polypeptide encodes a variable region, which when part of an Fab' or F(ab')<sub>2</sub> molecule, is reactive with T cells.
5. The molecule of any of claims 1, 2, 3, or 4 wherein the Fd polypeptide is chimeric.
6. A vector comprising the molecule of claim 1.
7. The vector of claim 6 which also encodes the light chain polypeptide of an immunoglobulin Fab' or F(ab')<sub>2</sub> fragment molecule.
8. A bacterium transformed with the vector of any of claims 6 or 7.

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9. A polynucleotide molecule comprising a yeast promoter sequence operably linked to a sequence encoding the Fd polypeptide of a Fab' or F(ab')<sub>2</sub> immunoglobulin fragment.
10. The molecule of claim 9 wherein the Fd sequence encodes two or more cysteine residues in the Fd hinge region on the carboxyl side of the disulfide bond formed between the light chain and the Fd chain.
11. The molecule of claim 9 wherein there are less than 30 amino acid residues between the cysteine residue with the highest amino acid number, or the cysteine residue closest to the carboxyl terminal, and the carboxyl terminal.
12. The molecule of claim 9 wherein the Fab' or F(ab')<sub>2</sub> molecule is reactive with T cells.
13. The molecule of any of claims 9, 10, 11, or 12 wherein the Fab' or F(ab')<sub>2</sub> molecule is chimeric.
14. A vector comprising the molecule of claim 9.
15. The vector of claim 14 which also encodes the light chain polypeptide of an immunoglobulin Fab' or F(ab')<sub>2</sub> fragment molecule.
16. A yeast transformed with the vector of any of claims 14 or 15.
17. A method for preparing an immunoglobulin Fab' molecule, comprising the steps of:

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- a) culturing a bacterium transformed with the vector of claim 7; and
- b) isolating the Fab' from the culture medium.

18. A method for preparing an immunoglobulin F(ab')<sub>2</sub> molecule, comprising the steps of:

- a) culturing a bacterium transformed with the vector of claim 7; and
- b) isolating the (Fab')<sub>2</sub> from the culture medium.

19. A method for preparing an immunoglobulin Fab' molecule, comprising the steps of:

- a) culturing a yeast transformed with the vector of claim 15; and
- b) isolating the Fab' from the culture medium.

20. A method for preparing an immunoglobulin F(ab')<sub>2</sub> molecule, comprising the steps of:

- a) culturing a yeast transformed with the vector of claim 15; and
- b) isolating the F(ab')<sub>2</sub> from the culture medium.

21. A method for preparing an immunoglobulin F(ab')<sub>2</sub> molecule, comprising the steps of:

- a) preparing a first Fab' by the method of claim 17 or claim 19;
- b) preparing a second Fab' by the method of claim 17 or claim 19; and
- c) combining the first Fab' and the second Fab' such that an F(ab')<sub>2</sub> is formed.

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22. A method of preparing an immunoconjugate comprising:
- a) preparing a Fab' by the method of either claim 17 or claim 19;
  - b) reducing the selectively reducible hinge cysteine of the Fab';
  - and
  - c) linking the reduced Fab' to a thiol-containing active moiety.

23. A method of preparing an immunoconjugate comprising:
- a) preparing a Fab' by the method of either claim 17 or claim 19;
  - b) reducing the selectively reducible hinge cysteine of the Fab';
  - c) linking the reduced Fab' to a thiol-containing activating compound; and
  - d) further linking the thiol-containing activating compound previously linked to reduced Fab' to a thiol-reactive active moiety.

24. The method of claim 23 wherein the activating compound is dithiobis(pyridine-N-oxide) or dithiobis(nitrobenzoate).

25. A method of preparing an immunoconjugate comprising:
- a) preparing a F(ab')<sub>2</sub> by the method of claim 18 or claim 20;
  - and
  - b) linking the F(ab')<sub>2</sub> to an active moiety.

26. A method of preparing an immunoconjugate comprising:
- a) preparing a F(ab')<sub>2</sub> by the method of claim 21; and
  - b) linking the F(ab')<sub>2</sub> to an active moiety.

27. A method for preparing Fab'-enzyme conjugate comprising:
- a) preparing a Fab' by the method of claim 17 or claim 19; and

- 64 -

- b) linking the Fab' to an enzyme.

28. The method for preparing the Fab'-enzyme conjugate of claim 27 wherein the enzyme is a ribosome-inactivating protein.

29. The method for preparing the Fab'-enzyme conjugate of claim 27 wherein the enzyme is ricin toxin A chain.

30. The method for preparing the Fab'-enzyme conjugate of claim 27 wherein the Fab' is specific for a human T cell.

31. The method for preparing the Fab'-enzyme conjugate of claim 27 wherein the Fab' is specific for CD5 or CD7.

32. A method for preparing F(ab')<sub>2</sub>-enzyme conjugate comprising:

- a) preparing an F(ab')<sub>2</sub> by the method of claim 18 or claim 20; and  
b) linking the F(ab')<sub>2</sub> to an enzyme.

33. The method for preparing the F(ab')<sub>2</sub>-enzyme conjugate of claim 32 wherein the enzyme is a ribosome-inactivating protein.

34. The method for preparing the F(ab')<sub>2</sub>-enzyme conjugate of claim 32 wherein the enzyme is ricin toxin A chain.

35. The method for preparing the F(ab')<sub>2</sub>-enzyme conjugate of claim 32 wherein the F(ab')<sub>2</sub> is specific for a human T cell.

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36. The method for producing the  $F(ab')_2$ -enzyme conjugate of claim 32 wherein the  $F(ab')_2$  is specific for CD5 or CD7.

37. A method for preparing  $F(ab')_2$ -enzyme conjugate comprising:

- a) preparing an  $F(ab')_2$  by the method of claim 21; and
- b) linking the  $F(ab')_2$  to an enzyme.

38. The method for preparing the  $F(ab')_2$ -enzyme conjugate of claim 37 wherein the enzyme is a ribosome-inactivating protein.

39. The method of preparing the  $F(ab')_2$ -enzyme conjugate of claim 37 wherein the enzyme is ricin toxin A chain.

40. The method for preparing the  $F(ab')_2$ -enzyme conjugate of claim 37 wherein the  $F(ab')_2$  is specific for a human T cell.

41. The method for producing the  $F(ab')_2$ -enzyme conjugate of claim 37 wherein the  $F(ab')_2$  is specific for CD5 or CD7.

42. An  $F(ab')_2$  with specificity for two different antigens.

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43. The  $F(ab')_2$  of claim 42 wherein said antigens are tumor-associated antigens.

44. A method of producing an  $F(ab')_2$  with specificity for two different antigens wherein said method comprises:

- a) preparing a first Fab' having a specificity for a first tumor-associated antigen by the method of claim 17 or claim 19;
- b) preparing a second Fab' having a specificity for a second tumor-associated antigen by the method of claim 17 or claim 19; and
- c) combining the first Fab' and the second Fab' such that an  $F(ab')_2$  with specificity for two different tumor-associated antigens is formed.

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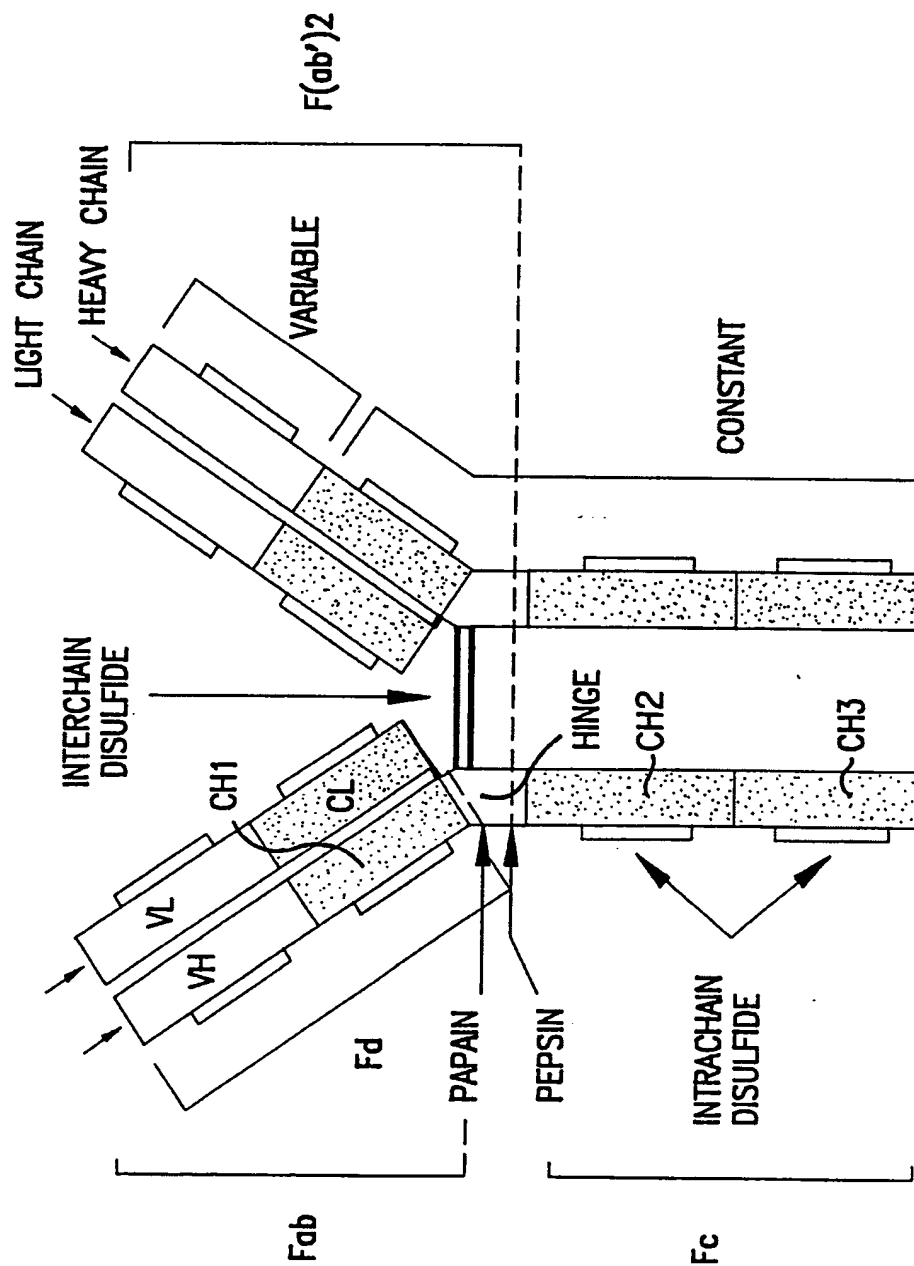
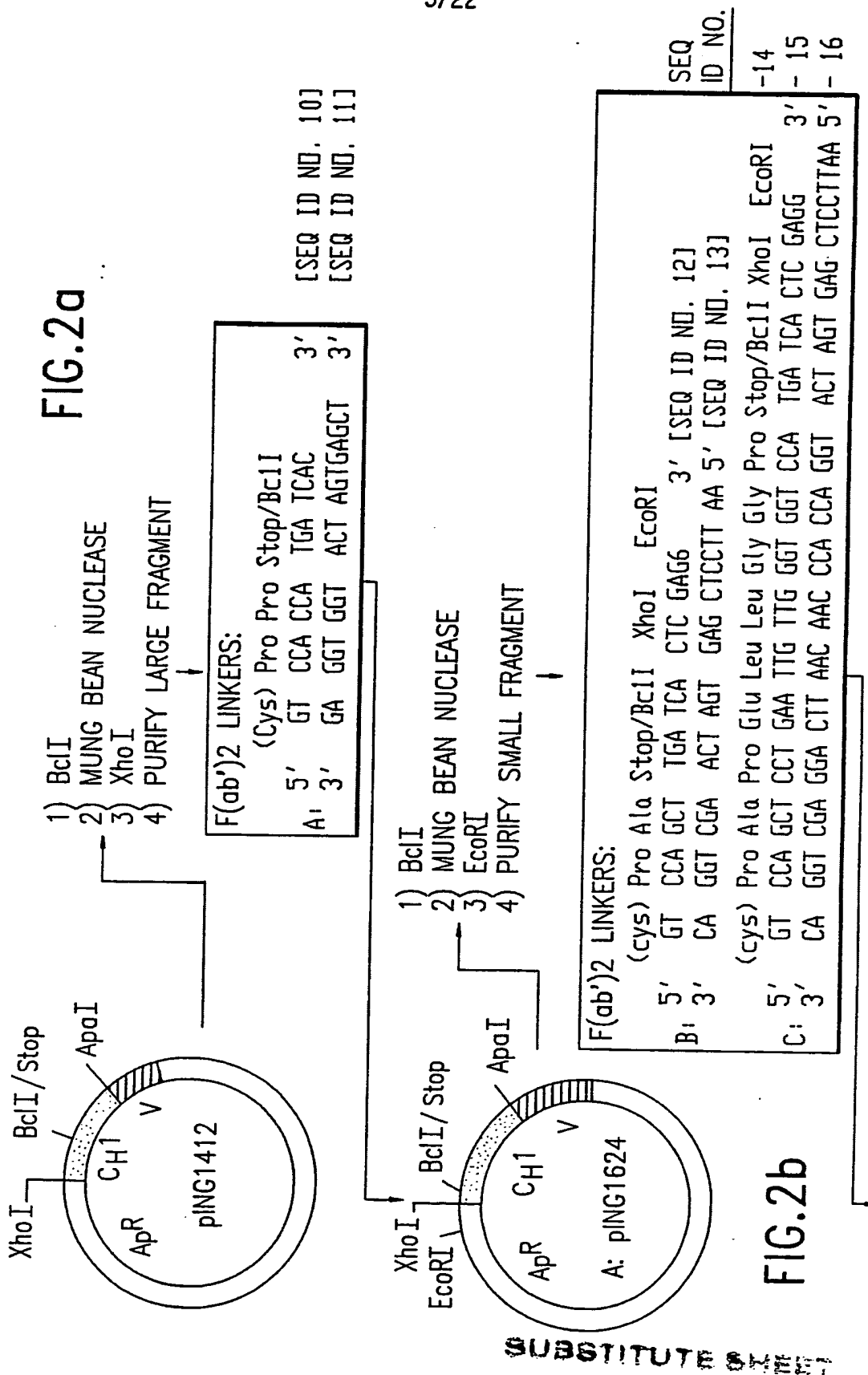


FIG.1a



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FIG.2c

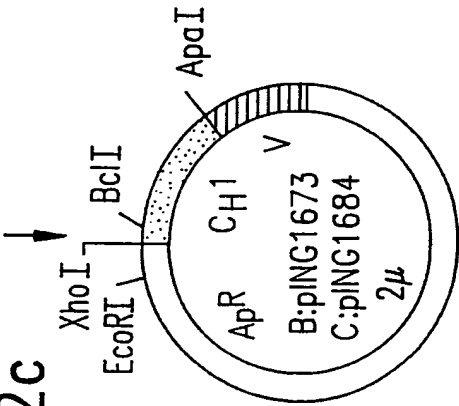
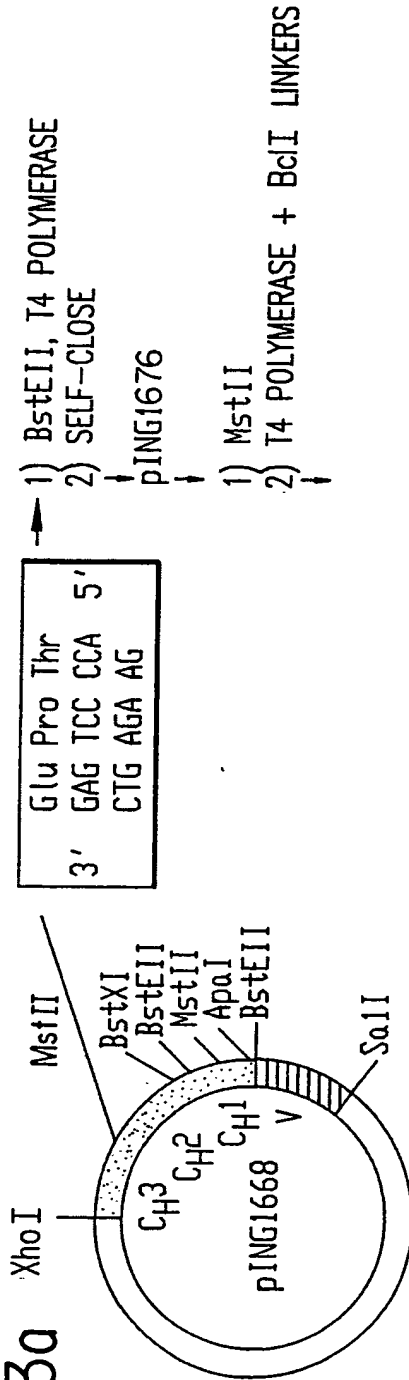
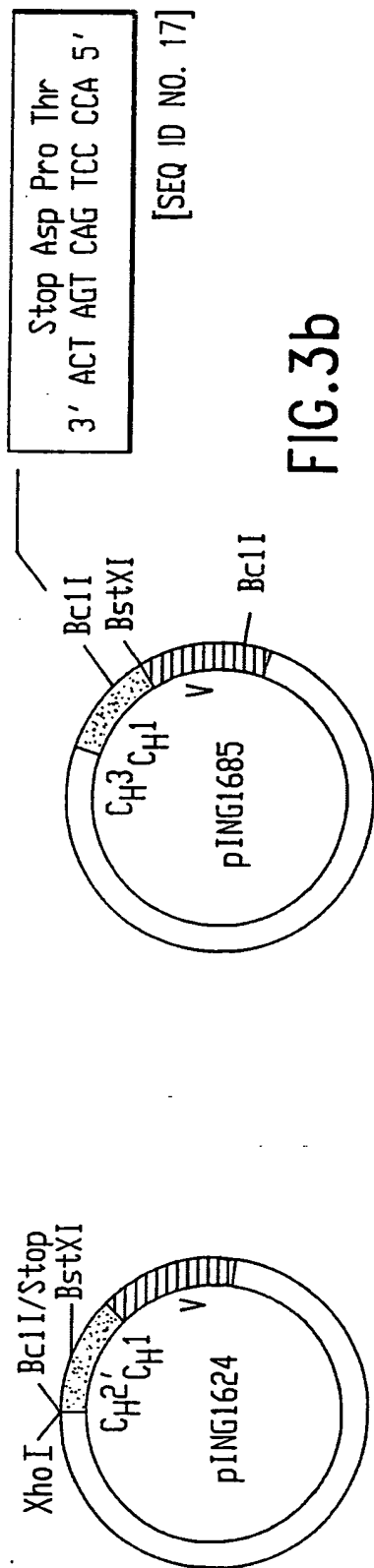


FIG.3a



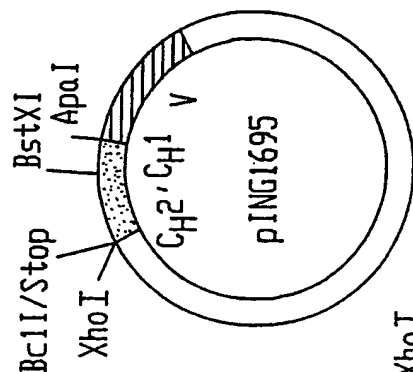
- 1) BstEII, T4 POLYMERASE  
2) SELF-CLOSE  
↓  
pING1676  
↓  
1) MstII  
2) T4 POLYMERASE + BclI LINKERS  
↓

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- 1) BclI + BstXI
- 2) PURIFY SMALL FRAGMENT

- 1) BclI + BstXI
- 2) PURIFY LARGE FRAGMENT



- 1) ApaI + XhoI
- 2) PURIFY SMALL FRAGMENT

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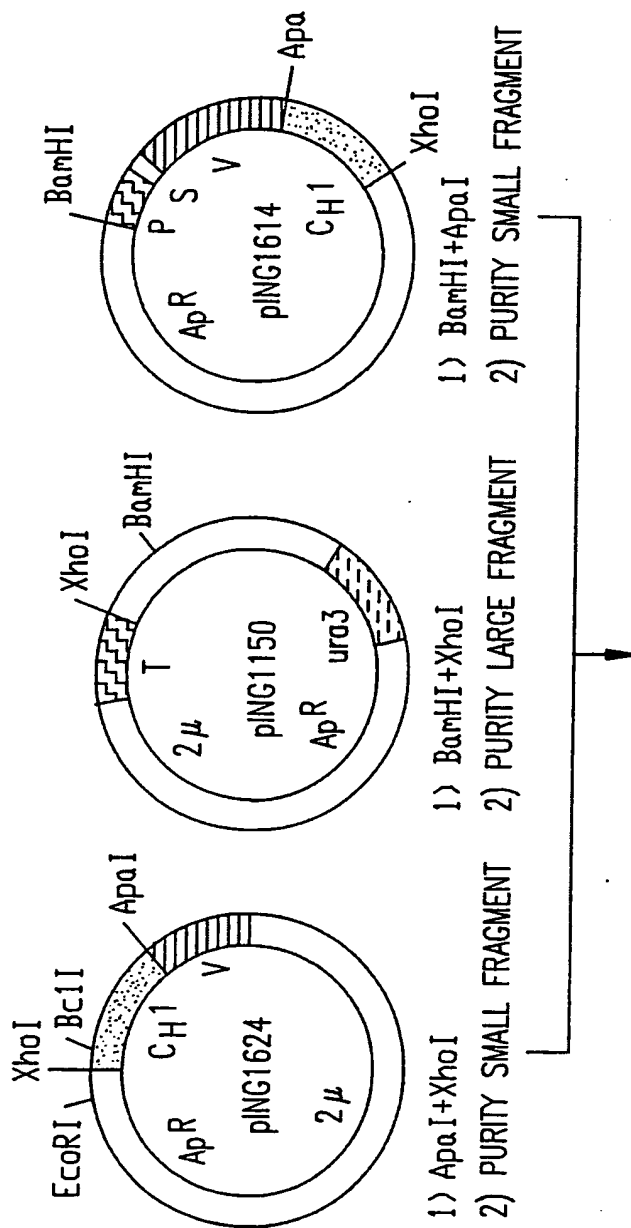
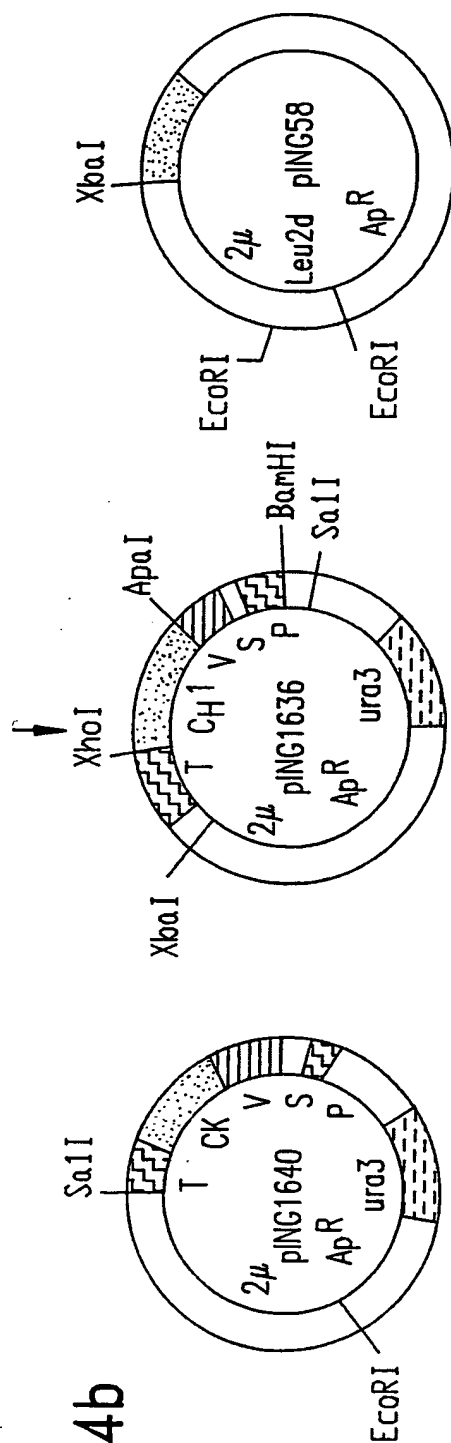


FIG.4a

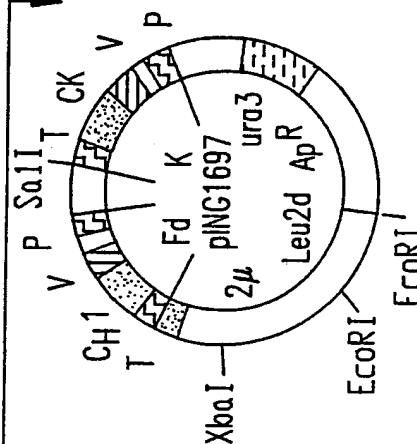
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- 1) Xba I + PARTIAL EcoRI
- 2) PURIFY 2 $\mu$ -Leu2d  
FRAGMENT

- 1)  $\text{SnI} + \text{XbaI}$
- 2) PURIFY  $\text{V}-\text{CH}_1$  FRAGMENT

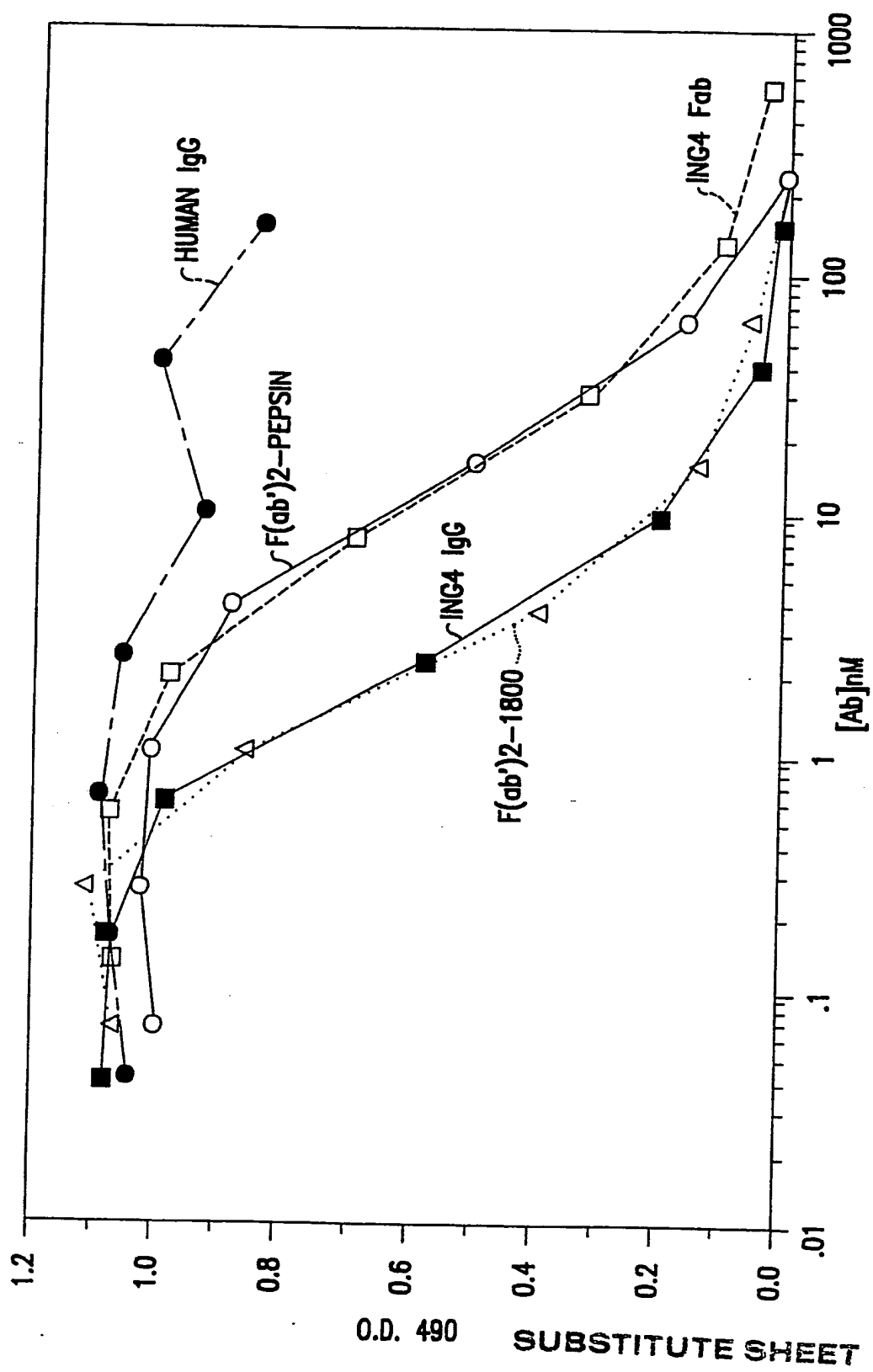
1)  $\text{SalI} + \text{EcoRI}$   
2) PURIFY *ura3* + V-CK  
FRAGMENT



**FIG. 4c**

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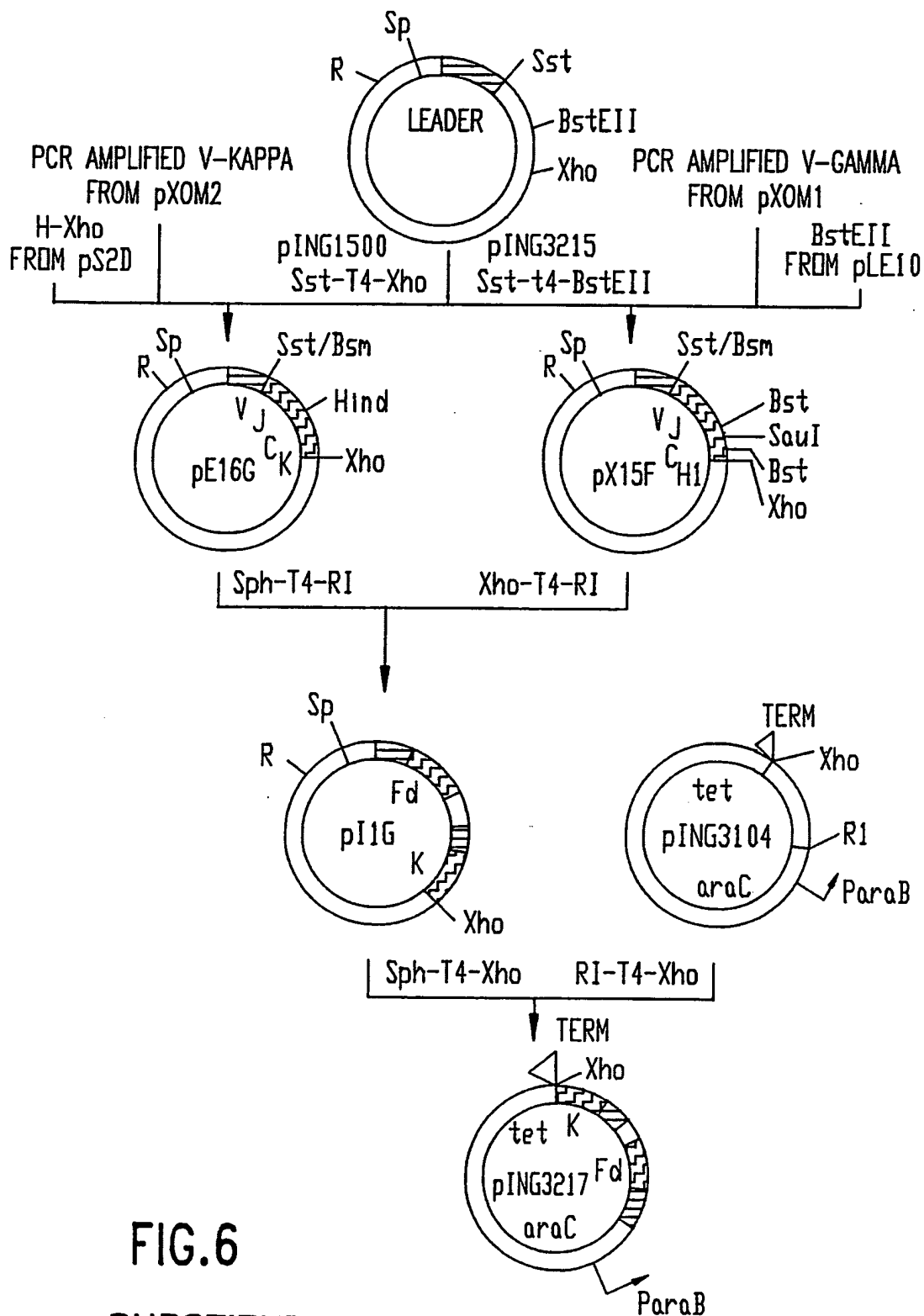


FIG.6

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# H-65 Fab Derivatives

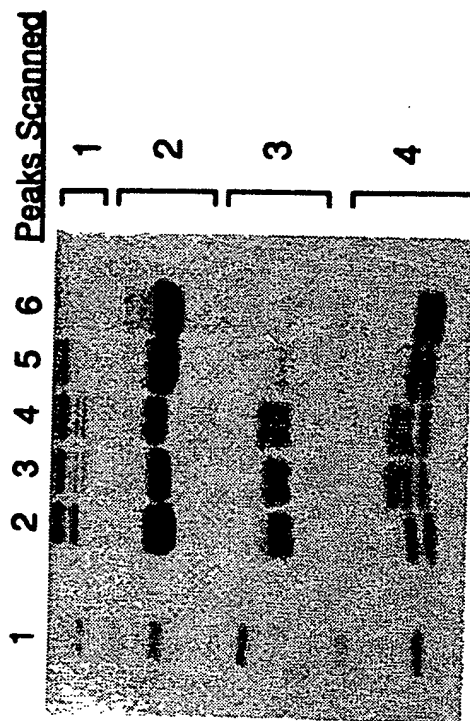


FIG. 7

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LANE#	DERIVATIVE	C-TERMINAL SEQUENCE	PLASMID
1	Bio-Rad standard		
2	Fab'	-CPP	pING3217
3	Fab'-S	-SPPC	pING3518
4	Fab'-S+9aa	-SPPCPAPELLGGP	pING3519
5	Fab'-2C+9aa	-CPPCPAPELLGGP	pING3219
6	Fab'-2C+9aa	- " "	pING3219

SEQ  
ID NO.

18- ATG GCT TGG GTG TGG ACC TTG CTA TTC CTG ATG GCA GCT GCC CAA - 45  
19- MET Ala Trp Val Trp Thr Leu Leu Phe Leu MET Ala Ala Gln - 15

L/M

AGT GCC CAA GCA CAG ATC CAG TTG GTG CAG TCT GGA CCT GAG CTG - 90  
Ser Ala Gln Ala Gln Ile Gln Leu Val Gln Ser Gly Pro Glu Leu - 30

AAG AAG CCT GGA GAG ACA GTC AAA ATC TCC TGC AAG GCT TCT GGG -135  
Lys Lys Pro Gly Glu Thr Val Lys Ile Ser Cys Lys Ala Ser Gly - 45

TAT ACC TTC ACA AAC TAT GGA ATG AAC TGG GTG AAG CAG GCT CCA -180  
Tyr Thr Phe Thr Asn Tyr Gly MET Asn Trp Val Lys Gln Ala Pro - 60

GGA AAG GGT TTA AGG TGG ATG GGC TGG ATA AAC ACC CAC ACT GGA -225  
Gly Lys Gly Leu Arg Trp MET Gly Trp Ile Asn Thr His Thr Gly - 75

FIG. 8a

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GAG CCA ACA TAT GCT GAT GAC TTC AAG GGA CGG TTT GCC TTC TCT -270  
Glu Pro Thr Tyr Ala Asp Asp Phe Lys Gly Arg Phe Ala Phe Ser - 90

TTG GAA ACG TCT GCC AGC ACT GCC TAT TTA CAG ATC AAC AAC CTC -315  
Leu Glu Thr Ser Ala Ser Thr Ala Tyr Leu Gln Ile Asn Asn Leu -105

AAA AAT GAG GAC ACG GCT ACA TAT TTC TGT ACA AGA CGG GGT TAC -360  
Lys Asn Glu Asp Thr Ala Thr Tyr Phe Cys Thr Arg Arg Gly Tyr -120

GAC TGG TAC TTC GAT GTC TGG GGC GCA GGG ACC ACG GTC ACC GTC -405  
Asp Trp Tyr Phe Asp Val Trp Gly Ala Gly Thr Thr Val Thr Val -135

J/CH

TCC TCA GCC  
Ser Ser Ala

FIG.8b

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SEQ  
ID NO.

20- ATG GAC ATG AGG ACC CCT GCT CAG TTT CTT GGA ATC TTG TTG CTC - 45  
21- MET Asp MET Arg Thr Pro Ala Gln Phe Leu Gly Ile Leu Leu Leu - 15

L/M

TGG TTT CCA GGT ATC AAA TGT GAC ATC AAG ATG ACC CAG TCT CCA - 90  
Trp Phe Pro Gly Ile Lys Cys Asp Ile Lys MET Thr Gln Ser Pro - 30

TCT TCC ATG TAT GCA TCT CTG GGA GAG AGA GTC ACT ATC ACT TGC -135  
Ser Ser MET Tyr Ala Ser Leu Gly Glu Arg Val Thr Ile Thr Cys - 45

AAG GCG AGT CAG GAC ATT AAT AGC TAT TTA AGC TGG TTC CAG CAG -180  
Lys Ala Ser Gln Asp Ile Asn Ser Tyr Leu Ser Trp Phe Gln Gln - 60

FIG. 9a

SCS INSTITUTE OF TECHNOLOGY

AAA CCA GGG AAA TCT CCT AAG ACC CTG ATC TAT CGT GCA AAC AGA -225  
 Lys Pro Gly Lys Ser Pro Lys Thr Leu Ile Tyr Arg Ala Asn Arg - 75

TTG GTA GAT GGG GTC CCA TCA AGG TTC AGT GGC AGT GGA TCT GGG -270  
 Leu Val Asp Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly - 90

CAA GAT TAT TCA CTC ACC ATC AGC AGC CTG GAC TAT GAA GAT ATG -315  
 Gln Asp Tyr Ser Leu Thr Ile Ser Ser Leu Asp Tyr Glu Asp MET -105

GGA ATT TAT TAT TGT CAA CAG CAG TAT GAT GAG TCT CCG TGG ACG TTC -360  
 Gly Ile Tyr Tyr Cys Gln Gln Tyr Asp Glu Ser Pro Trp Thr Phe -120

GGT GGA GGC ACC AAG CTG GAA ATC AAA C J/Ck -405  
 Gly Gly Gly Thr Lys Leu Glu Ile Lys -135

FIG.9b

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SEQ  
ID NO.

22- ATG GAG TCA GAC ACA CTC CTG CTA TGG GTG CTG CTC TGG GTT - 45  
23- MET Glu Ser Asp Thr Leu Leu Leu Trp Val Leu Leu Trp Val - 15

L / M

CCA GGC TCC ACT GGT GAC ATT GTG CTC ACC CAA TCT CCA GCT TCT - 90  
Pro Gly Ser Thr Gly Asp Ile Val Leu Thr Gln Ser Pro Ala Ser - 30

TTG GCT GTG TCT CTG GGG CAG AGA GCC ACC ACC ATC TCC TGC AGA GCC -135  
Leu Ala Val Ser Leu Gly Gln Arg Ala Thr Ile Ser Cys Arg Ala - 45

AGT GAA AGT GTT GAA TAT TAT GGC ACA AGT TTA ATG CAG TGG TAC -180  
Ser Glu Ser Val Glu Tyr Tyr Gly Thr Ser Leu MET Gln Trp Tyr - 60

FIG.10a

CAA CAG AAA CCA GGA CAG CCA CCC AAA CTC CTC ATC TAT GCT GCA	-225
Gln Gln Lys Pro Gly Gln Pro Pro Lys Leu Ile Tyr Ala Ala	- 75
TCC AAC GTA GAA TCT GGG GTC CCT GCC AGG TTT AGT GGC AGT GGG	-270
Ser Asn Val Glu Ser Gly Val Pro Ala Arg Phe Ser Gly Ser Gly	- 90
TCT GGG ACA GAC TTC AGC CTC AAC ATC CAT CCT GTG GGG GAG GAG	-315
Ser Gly Thr Asp Phe Ser Leu Asn Ile His Pro Val Gly Glu Glu	-105
GAT ATT GCG ATG TAT TTC TGT CAG CAG AGT AGG AAG GTT CCT TGG	-360
Asp Ile Ala MET Tyr Phe Cys Gln Gln Ser Arg Lys Val Pro Trp	-120
ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA	-405
Thr Phe Gly Gly Thr Lys Leu Glu Ile Lys	-135

J/Ck

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FIG.10b

SEQ  
ID NO.

24- ATG AAC TTC GGG CTC AGC TTG ATT TTC CTT GTC CTT GTT TTA AAA - 45  
25- MET Asn Phe Gly Leu Ser Leu Ile Phe Leu Val Leu Lys - 15

L/M

GGT GTC CAG TGT GAA GTG CAG CTG GTG GAG TCT GGG GGA GGC TTA - 90  
Gly Val Gln Cys Glu Val Gln Leu Val Glu Ser Gly Gly Leu - 30

GTG AAG CCT GGA GGG TCC CTG AAA CTC TCC TGT GCA GCC TCT GGA -135  
Val Lys Pro Gly Gly Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly - 45

TTC ACT TTC AGT GAC TTT TAC ATG TAT TGG GTT CGC CAG ACT CCG -180  
Phe Thr Phe Ser Asp Phe Tyr MET Tyr Trp Val Arg Gln Thr Pro - 60

GAA AAG AGG CTG GAG TGG GTC GCA ACC ATT AGT GAT GGT GGT ATT -225  
Glu Lys Arg Leu Glu Trp Val Ala Thr Ile Ser Asp Gly Gly Ile - 75

FIG.11a

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TAC ACC TAC TAT TCA GAC AGT GTG ATG GGG CGA TTC ACC ATC TCC -270-  
 Tyr Thr Tyr Tyr Ser Asp Ser Val MET Gly Arg Phe Thr Ile Ser - 90

AGA GAC AAT GCC AAG AAC AAC CTG TAC CTG CAA ATA AGC AGT CTG -315  
 Arg Asp Asn Ala Lys Asn Asn Leu Tyr Tyr Leu Gln Ile Ser Ser Leu -105

AAG TCT GAG GAC ACA GCC ATG TAT TAC TGT GCA AGA GAT CCC TAT -360  
 Lys Ser Glu Asp Thr Ala MET Tyr Tyr Cys Ala Arg Asp Pro Tyr -120

TCC TAC GAT AGT AGC CCC GCC TGG TTT GCT TAC TGG GGC CAA GGG -405  
 Ser Tyr Asp Ser Ser Pro Ala Trp Phe Ala Tyr Trp Gly Gln Gly -135

J/CH

ACT CTG GTC ACT GTC TCT GCA  
 Thr Leu Val Thr Val Ser Ala

-450  
 -150

FIG.11b

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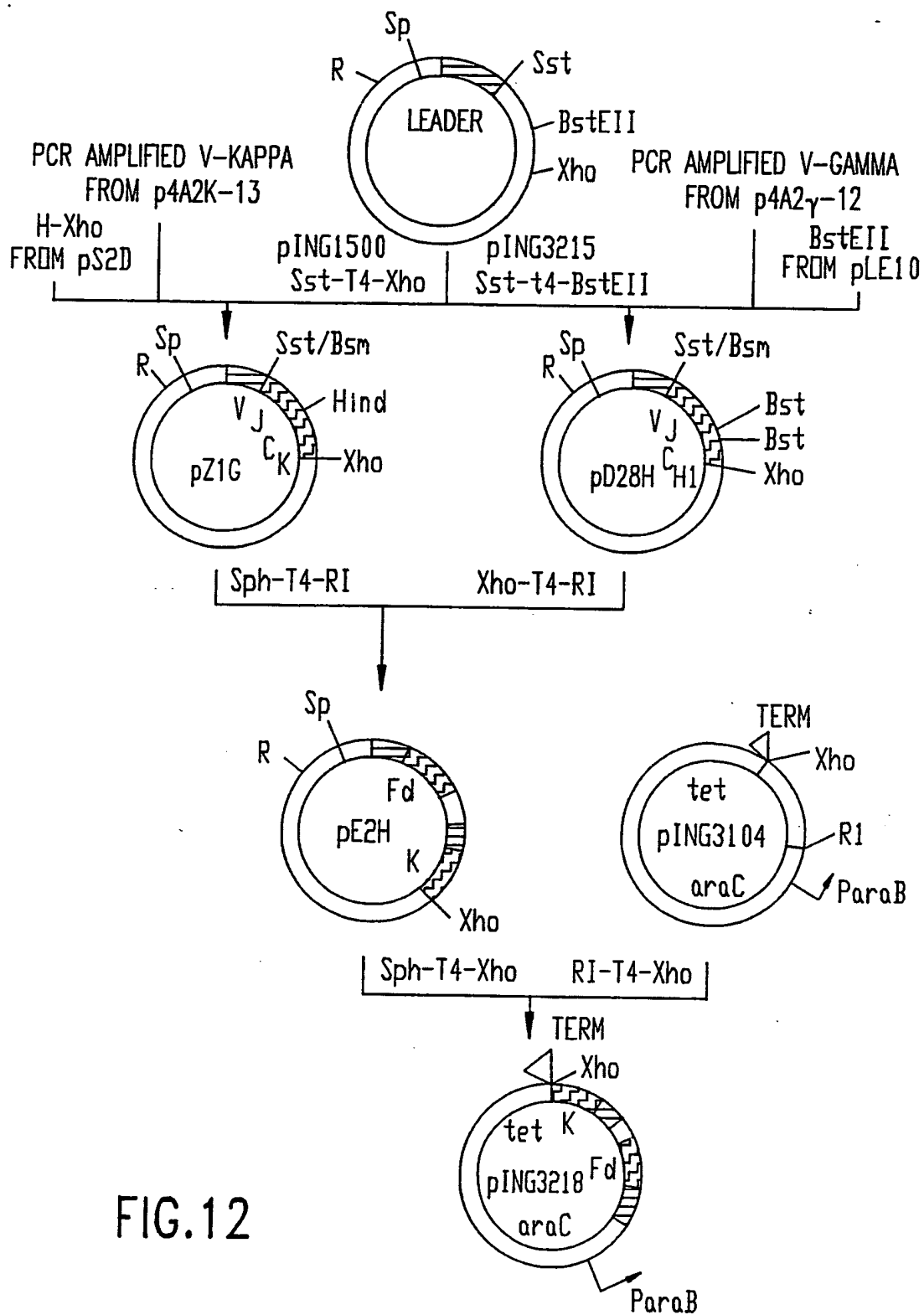


FIG.12

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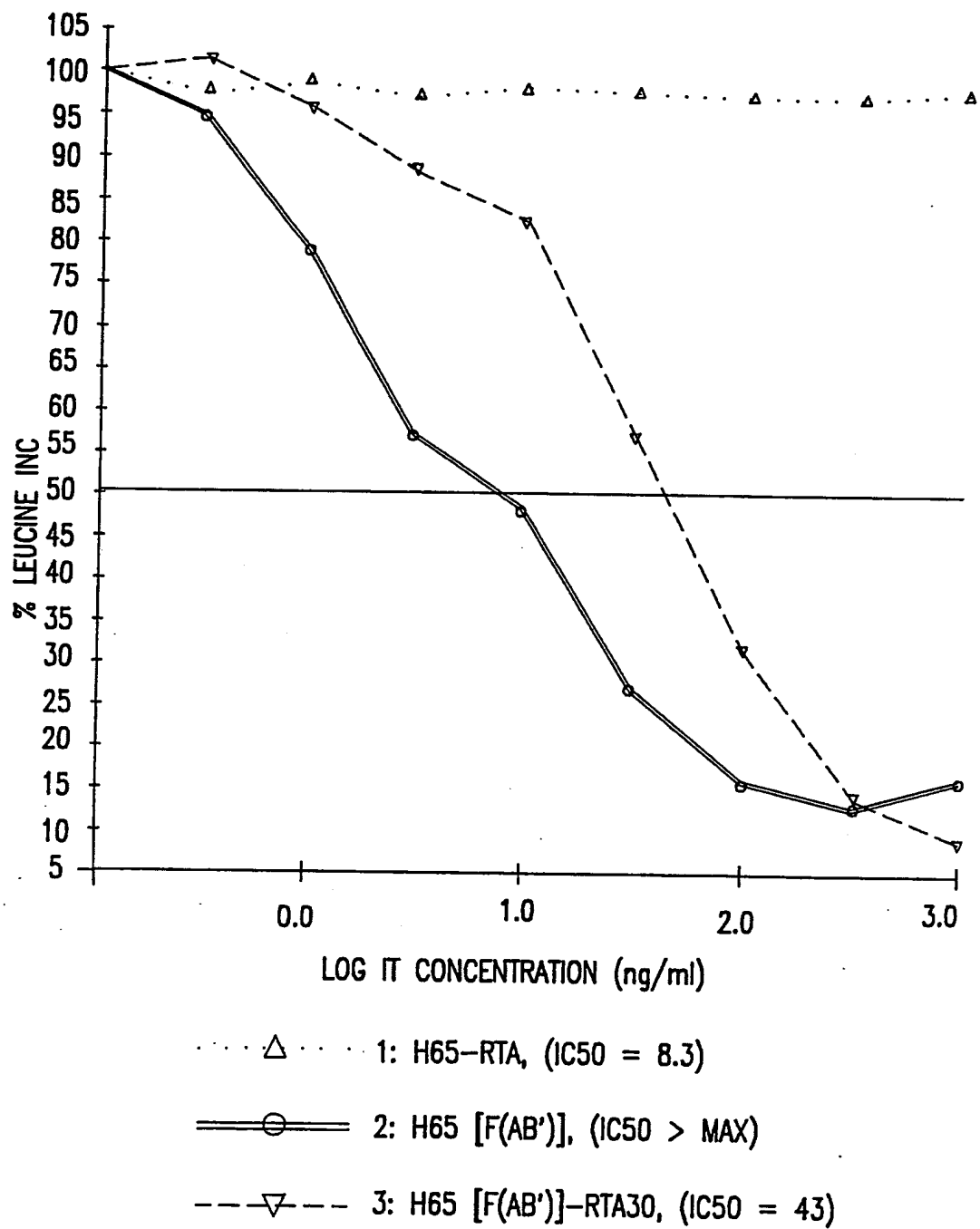
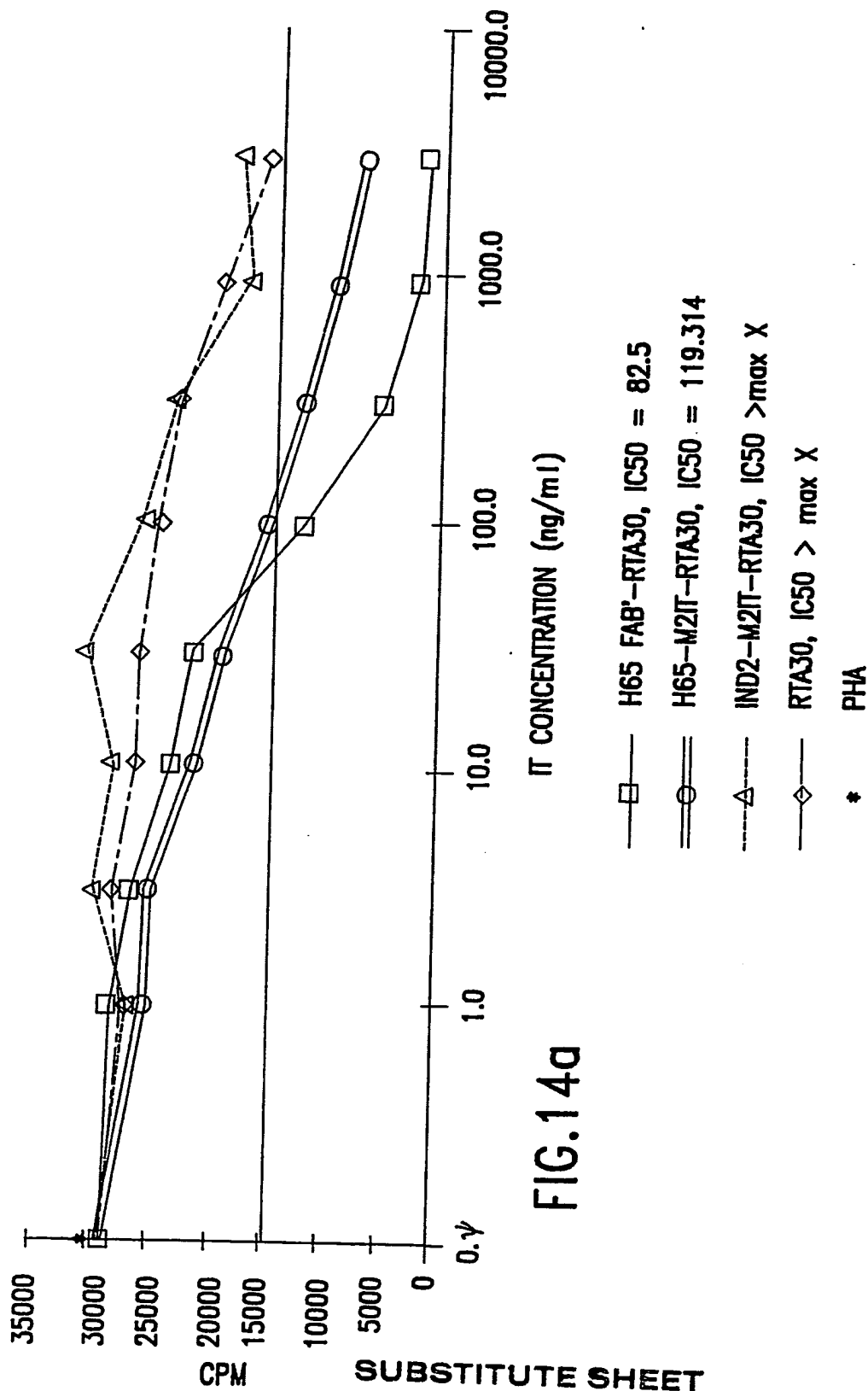


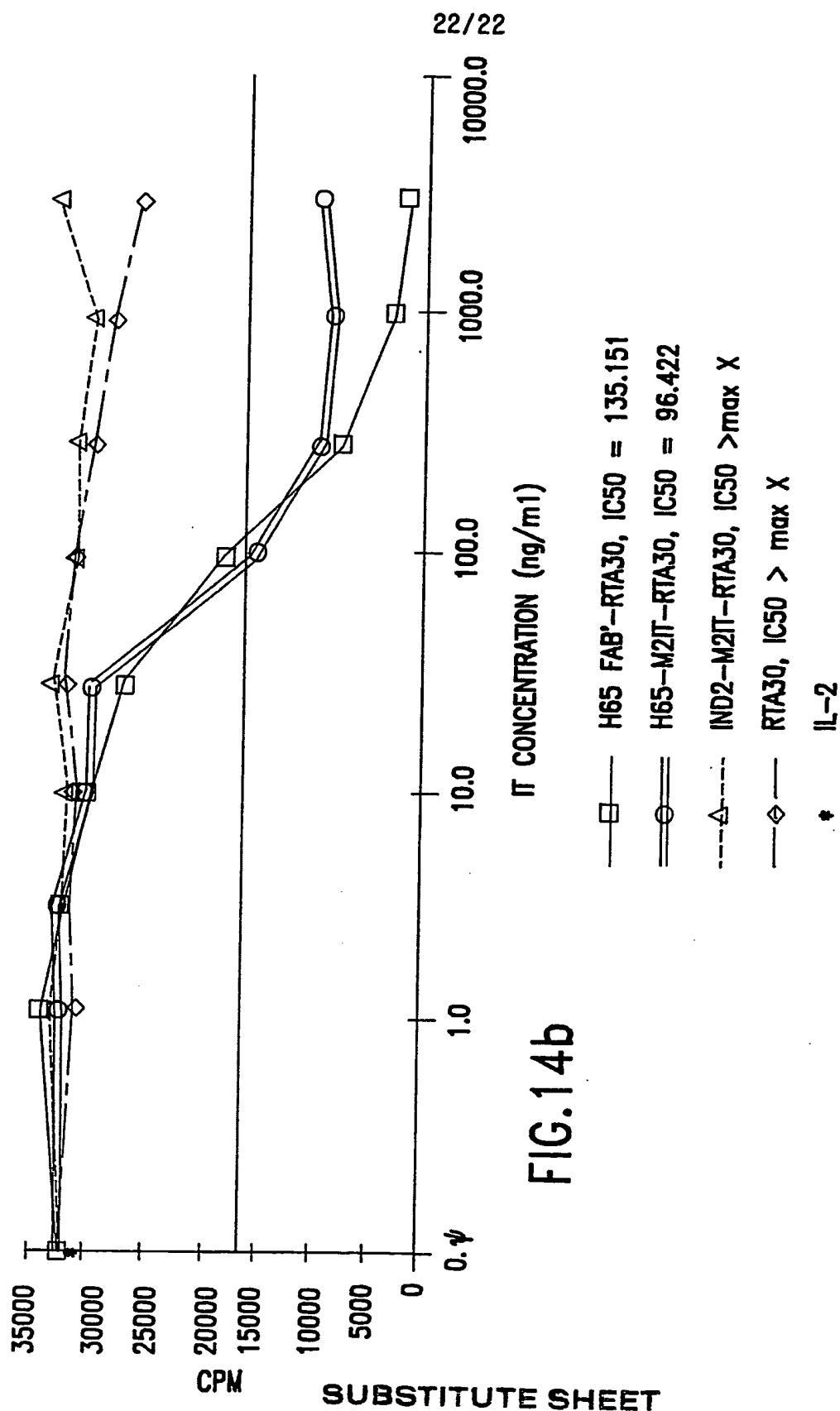
FIG.13

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/04976

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.8, 85.91; 435/69.6, 240.27, 320.1, 822, 848; 530/387.1, 388.1, 389.1, 391.5, 825, 867; 935/15

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

AUTOMATED PATENT SYSTEM, FILE:USPAT

DIALOG, FILE:5,55,155,399,350,73

KEYWORDS: ANTIBOD?, CYSTEIN?, SUBSTUT?, VARIANT?

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCIENCE, VOL. 240, ISSUED 20 MAY 1988, BETTER ET AL., "ESCHERICIA COLI SECRETION OF AN ACTIVE CHIMERIC ANTIBODY FRAGMENT", PAGES 1041-1043, SEE ENTIRE DOCUMENT.	1-44
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, VOL. 85, ISSUED NOVEMBER 1988, HORWITZ ET AL., "SECRETION OF FUNCTIONAL ANTIBODY AND Fab FRAGMENT FROM YEAST CELLS", PAGES 8678-8682, SEE ENTIRE DOCUMENT.	1-44
Y	PROTEIN ENGINEERING, VOL. 3, NO. 8, ISSUED 1990, LYONS ET AL., "SITE-SPECIFIC ATTACHMENT TO RECOMBINANT ANTIBODIES VIA INTRODUCED SURFACE CYSTEINE RESIDUES", PAGES 703-708, SEE ENTIRE DOCUMENT.	1-44



Further documents are listed in the continuation of Box C.



See patent family annex.

Special categories of cited documents:	
*A* document defining the general state of the art which is not considered to be part of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*E* earlier document published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*O* document referring to an oral disclosure, use, exhibition or other means	*Z* document member of the same patent family
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

28 September 1992

Date of mailing of the international search report

16 OCT 1992

Name and mailing address of the ISA/  
Commissioner of Patents and Trademarks  
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**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US92/04976

**A. CLASSIFICATION OF SUBJECT MATTER:**  
IPC (5):

A 61K 39/00, 35/14; C12P 21/06; C12N 5/00, 15/00, 1/00, 1/20; C07K 3/00, 13/00, 15/00

**A. CLASSIFICATION OF SUBJECT MATTER:**  
US CL :

424/85.8, 85.91; 435/69.6, 240.27, 320.1, 822, 848; 530/387.1, 388.1, 389.1, 391.5, 825, 867; 935/15

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